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Investigation of dietary zinc and linoleic acid interactions in the Sprague-Dawley rat

McCarthy, Patrick Vincent, Ph.D.

The University of North Carolina at Greensboro, 1988

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INVESTIGATION OF DIETARY ZINC AND LINOLEIC ACID INTERACTIONS IN THE SPRAGUE-DAWLEY RAT

by

Patrick Vincent McCarthy

A Dissertation Submitted to
the Faculty of the Graduated School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Greensboro
1988

Approved by

Dissertation Advisor

APPROVAL PAGE

This Dissertation has been approved by the following committee of the Faculty of the Graduate School at the University of North Carolina at Greensboro.

Dissertation Advisor: Helen C. Magee

Committee Members: Grace S. Kissling

Kenneth R. McCoy

Christine C. Gregory

November 3, 1988
Date of Acceptance by Committee

November 3, 1988
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The purpose of this study was to investigate the hypothesis of a dietary interaction in young rats fed diets with and without zinc and linoleic acid. The parameters used to assess the interaction(s) included food intake; weight gain; leukocyte alkaline phosphatase; bleeding time; appearance/muscle tone; dermal lesions; plasma zinc; plasma fatty acid profile; the testicular mineral concentrations of zinc, copper, iron, and manganese, and the total number of circulating leukocytes, granulocytes, and platelets in the animals fed the experimental diets. A randomized block design, based on initial body weights, involving a 2^2 factorial treatment arrangement was used in this study. Factors included two levels of zinc (1.5 ppm and 40 ppm) and two levels of linoleic acid (0% and 2% of total dietary calories). The experimental phase consisted of 4 test diets which were fed to 24 rats for 33 days. Data were analyzed using fixed effects repeated measures (RMANOVA), multivariate (MANOVA), or univariate (ANOVA) analysis of variance models. When either RMANOVA or MANOVA was employed, Roy's Maximum Root Criterion was used to test the hypothesis that the diets had no overall effect on the observed results.

RMANOVA has been used to answer two general questions in this investigation. The first question asks if there was any systematic change in the dependent variable group mean vectors over time. The second question addressed by RMANOVA asks if the response of the dependent variables differed depending upon both the diet and the point in time, during the experimental phase, in which data was collected.

MANOVA was used to determine if the response variables differed depending upon the assigned level of the diet.

The results of this study support the hypothesis that dietary zinc and linoleic acid interact in the laboratory rat. This investigation has identified growth, testicular manganese, platelet count, and the plasma fatty acid profile as physiological parameters that respond to a zinc X linoleic acid interaction. In addition, the time element associated with the onset of a dietary interaction in weight change and platelet count has been approximated.

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* * * * * * *

This dissertation is dedicated to Darlene A. McCarthy, my anima.

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CHAPTER I

INTRODUCTION

An interaction between dietary zinc and linoleic acid based on the individual and combined effects of dietary nutrient deficiencies over time has been proposed (Bettger, Reeves, Moscatelli, Reynolds & O'Dell, 1979). Zinc has been linked to cyclic metabolic patterns involving food intake (Krammer, Briske-Anderson, Johnson, & Holman, 1984) and there is the possibility that such patterns could be responsible for the alterations in enzyme activity and growth observed by Swenerton and Hurley (1968). The severity of a zinc deficiency is related to both degree and duration of inadequate zinc intake. The onset of linoleic acid deficiency signs is delayed when compared to signs associated with zinc deficiency but at some point in time the effects of each deficiency state on a physiological function theoretically may converge. One possible relationship between these two nutrients is shown in Figure 1.

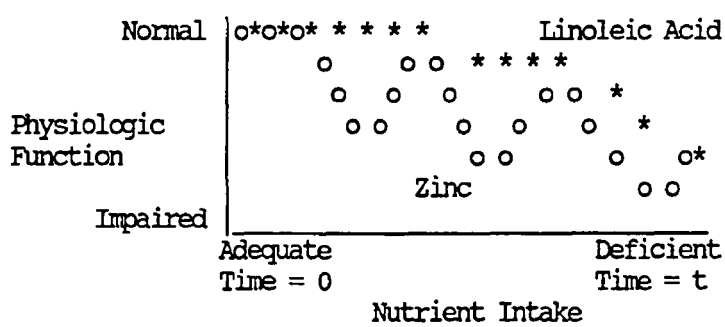

Figure 1. Possible Relationship of Zinc and Linoleic Acid Over Time to Physiologic Function as Dietary Deficiency Progresses.

Zinc must be consumed daily in order to maintain the functional integrity of basic enzymatic processes involved in nucleic acid and protein synthesis which regulate cell division, growth, and repair (Sandstead & Evens, 1984). When zinc is withheld from the diet of a weanling rat, the experimental effects can be detected within hours, and death will follow within a matter of weeks. Based on the observation that 24 hours after consuming a zinc deficient diet the plasma zinc concentration of weanling rats decreased by 55%, Dreosti, Tao, and Hurley (1968) suggested that physiologic zinc deficiency occurs in rats before any significant depletion of body zinc stores can take place.

The onset of signs and symptoms that result from withholding dietary lipid are less abrupt, and rats can survive on such a diet for months (Burr, Burr, & Miller, 1932). The metabolism of essential fatty acids depends on the activity of several different desaturase and elongase enzymes. Experiments involving essential fatty acid (EFA) deficiency in the rat have shown that the time necessary to produce a deficiency is decreased if the diet is also deficient in zinc (Cunnane, Huang, Horrobin, & Davignon, 1981). Kramer et al., (1984), have reported that the effect of zinc on polyunsaturated fatty acid profiles was related to reduced food intake secondary to zinc deficiency. Ayala and Brenner (1987), however, suggested that zinc participates in a critical desaturase reaction in the conversion of linoleic acid to arachidonic acid.

Bettger et al. (1979) speculated that since the signs of zinc deficiency and EFA deficiency were similar, zinc and the essential fatty acids interact metabolically in the rat. Many nutritional studies support the conclusion that dietary zinc and EFA interact metabolically (Cunnane & Wahle, 1981; Huang, Cunnane, Horrobin, & Davignon, 1982). However, the exact nature of a zinc and linoleic acid interaction and the minimum time necessary to establish an interacting zinc and linoleic acid deficiency in the laboratory rat have not been established.

Rationale of Study

A repeated measures experiment was planned to help identify possible sites of, and the minimum time necessary to produce an interacting zinc and linoleic acid deficiency in the Sprague-Dawley rat. The purpose of this study was to investigate the hypothesis of a dietary interaction by observing the number, volume, concentration or appearance of selected physiological variables and characteristics in young rats fed diets with and without zinc and linoleic acid. The criteria used to assess the interaction(s) included: food intake; weight gain; dermal lesions; appearance/muscle tone; leukocyte alkaline phosphatase; bleeding time; total leukocyte count; absolute granulocyte count; platelet count; mean platelet volume; plasma zinc concentration; plasma fatty acid profile; and the testicular concentrations of zinc, copper, iron, and manganese. The results of this experiment should help to clarify the dietary effects of zinc and linoleic acid that occur over time.

Limitations of Study

The major limitation in this type of repeated measures study involving hemic variables and the laboratory rat is the difficulty associated with obtaining a good quality blood sample in sufficient quantity in a routine non-traumatic manner. The total blood volume in the rat contributes approximately 10% of the body weight, and samples are generally collected by cardiac puncture, capillary collection from the orbital sinus, or by tail puncture, incision, or snip (Mitruka & Rawnsley, 1981). The method of tail snip was selected because preliminary experimental results indicated a sufficient volume of blood could be obtained repeatedly in this manner. This decision was made even though it was known that the tail of the rat is often severely affected by nutritional deficiencies. Other methods of blood collection were eliminated because of the risk of killing the animals, introducing bacterial infection, or the lack of reliability in obtaining sufficient blood quantity.

CHAPTER II

REVIEW OF LITERATURE

Zinc and Essential Fatty Acid Requirements and Deficiency Signs

Zinc and linoleic acid are essential dietary nutrients for the laboratory rat and male weanling rats fed an egg white protein based diet must consume at least 12 parts per million (ppm) zinc per day in order to experience maximum body weight gain (Forbes & Yohe, 1960). A higher minimum zinc consumption (approximately 100 ppm per day) is required to support optimal reproductive performance (Swenerton & Hurley, 1968). Signs of zinc deficiency which can be produced within 28 days in weanling rats fed a diet containing approximately 2 ppm zinc include growth retardation; immature hair coats; fissures at the corners of the mouth; scaly feet; alopecia; dermal lesions; emaciation; edema around the eyes, mouth, and feet; a "kangaroo-like" posture,and death (Swenerton & Hurley, 1968). A partial listing of other sequela associated with consumption of a zinc deficient diet include a decrease in plasma zinc concentration, an increase in the number of circulating polymorphonuclear neutrophils (Dreosti, Shyy-Haw, & Hurley, 1968), decreased enzymatic activity of intestinal alkaline phosphatase, alcohol dehydrogenase and glutamic dehydrogenase (Kfoury, Reinhold, & Simonian, 1968), and reproductive disorders involving the testis and estrous cycle (Swenerton & Hurley, 1968). Cunnane, Horrobin, and Manku

(1984) also reported that zinc deficient rats have lower testicle, thymus, and epididymal fat pad organ weights but normal liver and adrenal gland weights when compared to controls animals fed a zinc adequate diet.

The requirement for linoleic acid is proportional to the energy supplied by the diet and it has been estimated that linoleic acid should supply between 1% and 3% of the dietary energy (Hassam, Rivers, & Crawford, 1977). A report by the National Academy of Science (1978), based on a review of the literature, placed the linoleic acid requirement for male rats at approximately 1.3% of the dietary energy, although, the percentage may increase if saturated fatty acids contribute more than 5% of the diet by weight. In an investigation of the nutritional and metabolic interrelationships between fatty acids, Holman (1964) found that weight gain decreased if saturated fatty acids were increased while linoleate was held constant in the diet and concluded that saturated and unsaturated fatty acids interact in tissues. Holman (1971), also suggested that the increased requirement for linoleic acid in a diet containing high amounts of saturated fat may reflect the involvement of essential fatty acids in saturated fatty acid metabolism.

Essential fatty acids have been associated with cell membrane integrity, intercellular matrix integrity, water retention, weight gain, and membrane viscosity. These acids also exert stearic influences on enzymes, and serve as precursors of prostaglandins. Rats consuming a diet deficient in essential fatty acids will exhibit

diminished growth; a scaly dermatitis on the paws and tail which is obvious after consuming the deficient diet for approximately two weeks; loss of muscle tone, and microscopic changes in the skin, kidneys, ovaries, and testis (Holman, 1971).

Based on the similarities of the signs that develop when either zinc or essential fatty acids are deficient in the diet of a weanling rat (decreased rate of growth, skin lesions, and reproductive disorders) Bettger et al. (1979) proposed that these nutrients interact metabolically. Several different approaches have been used to study the effects of essential fatty acids in zinc deficient rats including: EFA supplementation, deficiency, metabolic, and composition studies (Cunnane & Horrobin, 1985). Evidence for a metabolic interaction between these nutrients includes a report that zinc is involved in the enzymatic conversion of linoleic acid to arachidonic acid (Horrobin & Cunnane, 1980). A decrease in the activity of delta-6 desaturase may be the primary biochemical lesion responsible for the growth retardation and teratogenesis associated with zinc deficiency (Dresoti, Manual, Russell, & Buckley, 1985).

Role of Zinc in Essential Fatty Acid Metabolism

Zinc may be a cofactor of delta-6 desaturase, an enzyme that participates in the conversion of linoleic acid to gamma-linolenic acid (Huang, et al., 1982; Ayala & Brenner, 1983). Brenner (1981) reported that availability of delta-6 desaturase is rate limiting in this conversion. Ayala & Brenner (1987) found that a dietary zinc deficiency was associated with decreased rates of linoleic acid

conversion to higher homologues and concluded that in zinc deficiency the activities of desaturases and elongases involved in the metabolism of fatty acids were suppressed. Kramer et al. (1984), however, suggested reduced food intake and not zinc deficiency per se affected the delta-6-desaturase step in the metabolism of essential fatty acids. Fogerty, Ford, Dreosti, and Tinsley (1985), compared the results of several investigations which demonstrated that zinc deficiency, reduced food intake, type of tissue (mammary, testis, or liver), type of experimental study (fatty acid composition or enzyme activity) and lipid fraction (phospholipid or neutral lipid) apparently influence delta-6 desaturase activity and concluded that the role of zinc in fatty acid metabolism was still an enigma.

Bleeding Time and Platelets

Results of several reports have implicated zinc in the metabolism of essential fatty acids or their homologues which include the prostaglandins and thromboxanes. Linoleic acid is a precursor of the prostaglandins and thromboxane A_2 which participates in platelet aggregation (Willis, 1984). Since the concentrations of prostaglandins are decreased when zinc is deficient in the diet, Bettger et al. (1979), have suggested that zinc is involved in the conversion of arachidonic acid to either a prostaglandin precursor or derivative. In support of the hypothesis that zinc is involved in prostaglandin G_2 metabolism, O'Dell, Reynolds, and Reeves (1977) reported that bleeding time, a measure of the hemostatic function of skin and of the platelets capacity to respond to injury, was extended in zinc deficient rats.

Bleeding is prolonged when platelet aggregation is absent, weak, or when the platelet count is reduced. When the platelet count is normal, a long bleeding time suggests a platelet dysfunction. Zinc deficiency apparently has an effect similar to aspirin which prolongs bleeding time by inhibiting prostaglandin biosynthesis (O'Dell, et al. 1977). Platelets, which form the hemostatic plug that stops bleeding, contribute approximately 70% of the zinc found in the buffy coat of a blood sample (Milne, Ralston, & Wallwork, 1985). Chvapil (1976) previously reported that platelet aggregation was inhibited by zinc ions. There is the possibility that both zinc deficiency and excess affect bleeding time by either depressing prostaglandin synthesis from linoleic acid or by inhibiting platelet aggregation. Zinc has been reported to have different effects (enhance chemotaxis and inhibit phagocytosis in PMN cells) on functions within a single cell type (Prasad, 1982; p.50). The possibility that an interaction between zinc and linoleic acid will affect the bleeding time, the number of circulating platelets or the mean platelet volume has not been established.

Leukocyte Alkaline Phosphatase

The exact function of leukocyte alkaline phosphatase (LAP) is not known. The activity of LAP, a zinc metalloenzyme located in the plasma membrane of rat polymorphonuclear neutrophil (PMN) cells, is sensitive to the prostaglandin concentration in vitro (Das, 1983; Millard, Gerard, & Schneider, 1979). The concentration of this enzyme is apparently not sensitive to the intracellular zinc concentration

(Fredricks, Thanka, & Valentine, 1964), and the enzyme does appear to be more concentrated in mature PMN cells (Beisel, 1967). The zinc-activated form of leukocyte alkaline phosphatase is low in normal PMNs but is sometimes elevated in the presence of infection and stress situations (Beisel, 1967). LAP tends to increase for short durations after a fever or stress has peaked and the PMN count has decreased (Beisel, 1967).

Alkaline phosphatase has several iso-enzymatic forms that require zinc as a cofactor. The concentration of intestinal alkaline phosphatase apparently increases when fat, but not protein or carbohydrate, is increased in the diet (Kleerkoper, Horne, Cornish, & Posen, 1970). Although, both zinc and lipids (linoleic acid and prostaglandins) are involved in the metabolism of leukocyte alkaline phosphatase, the possibility that an interaction between zinc and linoleic acid will affect the concentration of this enzyme in PMN cells remains to be investigated.

Total Leukocyte and Polymorphonuclear Neutrophil Count

Zinc dietary deficiency is associated with a characteristic change in the normal leukocyte differential count in rats. Zinc deficiency apparently does not affect the total number of leukocytes in the circulation (approximately 12,500/mm^3 in 50 day old Sprague-Dawley rat) but will cause the number of circulating polymorphonuclear neutrophils (approximately 1,700/mm^3 in 50 day old S-D rat) to increase by about 40% (Davis, Wallwork, & Sandstead, 1984; Dreosti, Shyy-Hwa, & Hurley, 1968; Jain, 1986). The change in number of PMNs could be related to

their increased cellular activity (Wannamacher, Pekarek, Klainer, Bartelloni, Dupont, Hornick, & Beisel, 1975), to increased phagocytosis associated with depressed serum zinc values (Lennard, Bjornson, Petering, & Alexander, 1974) or to a normal physiological process related to maturation. Chvapil (1976), reported that as the plasma zinc concentration increases PMN cell motility, and phagocytosis will decrease.

The principal function of neutrophils is to fight bacterial infection, and PMNs account for approximately 93% of the circulating granulocytes in rat blood (Jain, 1986). Since PMNs account for the majority of granulocytes, the total granulocyte count recorded by a model S-PLUS Coulter Counter could substitute for a microscopic neutrophil count. Approximately 5% of the weight of normal neutrophils is contributed by lipid (Gottfried, 1972), but it is not known if an interaction between dietary zinc and linoleic acid will affect the total leukocyte, or the absolute polymorphonuclear neutrophil count.

Dermal Lesions

Some of the effects of zinc deficiency apparently can be ameliorated by administration of essential fatty acids. For instance, Clejan, Castro-Magana, Collipp, Jonass, and Maddaiah (1982) reported that zinc deficiency was associated with testicular atrophy which could be alleviated with arachidonic acid. Although linoleic acid can repair dermal lesions caused by zinc deficiency, gamma-linolenic acid (a product of linoleic acid desaturation and elongation) appears to have a more general role in reversing the effects of a zinc deficiency (Huang

et al.,1982). Ziboh and Hsia (1972), reported that the external application of prostaglandin E_2 to the skin of an EFA deficient rat would cure the dermal lesions associated with deficiency.

While discussing a correlation between dermatitis and growth in the essential fatty acid deficient rat, Holman (1964) speculated that a growing animal will deplete EFA reserves forming new cells faster than an animal whose growth is retarded. Therefore, the more growth the animal experiences the more severe will be the dermatitis. Prasad, Oberleas, Wolf, and Horwitz (1967) applied a similar rationale to growing cells in a zinc deficient rat and concluded that apoenzymes which normally combine with zinc become increasingly non-functional resulting in suppression of growth. While both zinc and EFA deficiencies will suppress cellular growth and result in dermal lesions the time course of interacting deficiency has not been established.

Appearance/Muscle Tone, Food Intake and Weight Gain

Hsu and Anthony (1975) reported that rats fed a zinc deficient diet experience an increased rate of muscle catabolism as evidenced by increased urinary excretion of nitrogen, urea, and uric acid. This muscle catabolism could be secondary to the hypophagia associated with zinc deficiency since zinc deficient rats will only consume about 10 grams food/day compared to 12-17 grams food/day on a zinc adequate diet (Bieri, Stoewsand, Briggs, Phillips, Woodard, & Knapka, 1977). A phenomenon similar to muscle catabolism has been described in essential fatty acid deficient rats. While discussing muscle tone, Holman (1971), described EFA deficient rats as having a "distinctly soft,

flaccid feeling when they are handled, in distinction to the firmness noted in normal rats". Burr et al. (1932), reported that when the diet of EFA deficient rats was supplemented with linoleic acid the animals gained weight, skin lesions cleared, and the muscle tone became noticeably better. The possibility that a zinc and linoleic acid interaction has an observable effect on appearance/muscle tone, food intake, or weight gain remains to be shown.

Plasma Lipids

Bettger et al. (1979) reported that zinc deficiency did not affect the plasma fatty acid profile, and Cunnane et al., (1984) reported that zinc deficiency did not alter the plasma palmitic acid concentration. Clejan et al. (1982), however, reported that a decrease in the plasma arachidonic acid concentration was associated with zinc deficiency. Chen (1979) found a substantial variation in the plasma palmitic acid concentration of rats fed free fatty acids. Feeding palmitic acid as the only dietary lipid source may result in depressed growth of rats (Alfin-Slater & Morris, 1965), and this depression may be a sign that can be attributed to essential fatty acid deficiency. The conversion of linoleic acid to arachidonic acid however, was not impaired if the diet contains only palmitic and linoleic acids (Mohrhaur & Holman, 1967). Palmitic acid can be elongated to form stearic, oleic, and eicosatrienoic acids or oxidized to form myristic acid and other shorter chain fatty acids.

When the diet is deficient in essential fatty acids, oleic acid will be desaturated and elongated to form eicosatrienoic acid (Fulco &

Mead, 1959). Supplementing an EFA deficient diet with linoleic acid however, will inhibit this conversion. (Mohrhaur & Holman, 1963). Based on the inhibitory relationship of linoleic acid on oleic acid desaturation and elongation, Holman (1960) developed the trienoic:tetraenoic fatty acid ratio as an index of essential fatty acid deficiency. When linoleic acid is the major polyunsaturated fatty acid in the diet, ratio values above 0.4 were associated with EFA deficiency (Holman, 1960). The trienoic:tetraenoic ratio reflects in vivo synthesis since neither of the fatty acids used in the ratio are common dietary components (Holman, 1964). Although, the validity of the ratio has been questioned (Huang et al., 1982), a superior index of EFA deficiency is not available at this time. The predictable consequences of zinc and essential fatty acids on the serum fatty acid profile have not been established.

Testicular Mineral Concentration

A zinc deficient diet has been linked to reproductive disorders, and Becker and Hoekstra (1968) suggested that such a diet will consistently and significantly reduce the testicular zinc concentration in rats. Lei, Abbasi, and Prasad (1976) found that the main effect of zinc on reproductive organs is at the testicular level and that zinc deficiency alters testicular steroidogenesis. Zinc deficiency results in testicular weight loss (Cunnane et al., 1984), and a rat must consume approximately 100 ppm zinc/day to insure against the changes (atrophy and spermatogenic arrest) that are associated with deficiency (Swenerton & Hurley, 1968). Prasad et al. (1967) investigated the

changes in testicular zinc, iron, copper, calcium, and magnesium that result from dietary zinc deficiency and concluded that in deficient animals the iron concentration will increase and replace zinc in the testis. In a more recent article Prasad (1982; p.49), suggested that zinc exerts a protective effect on sperm and other cells by decreasing their cellular activity and stabilizing their membranes. Roth and Kirchgessner (1977) reported that short-term zinc deficient diets will cause the manganese content of various tissues to increase. Leach and Lilburn (1978) found that manganese, a mineral that is essential for growth and reproduction was necessary for the synthesis of steroids in the testis.

The effect of EFA deficiency on reproduction has been recognized for a number of years. Burr and Burr (1930), reported on a sterility in male rats fed an EFA deficient diet that was characterized by loss of normal sex responses and an inability to sire normal litters. Apparently zinc deficiency will alter the concentration of several minerals in the testis and will also affect steroid synthesis and cell membrane stability. Whether an interaction of zinc and linoleic acid will alter the concentrations of copper, iron, or manganese in the testis of the rat remains to be determined.

Experimental Duration and Deficiency Signs

Excluding all sources of zinc from the diet will result in detectable signs and symptoms associated with dietary zinc deficiency within 24 hours and death within 28 days (Swenerton & Hurley, 1968). Burr and Burr (1930), however, demonstrated that a rat fed an essential

fatty acid free diet could survive up to 70 days. Thus experiments pertaining to zinc deficiency states tend to be short-term when compared to the duration of EFA deficiency experiments.

Bettger et al. (1979) found that a combined zinc and EFA deficiency would accentuate dermal lesions and depress the growth rate of rats after 49 days. Based on the exaggerated response of rats to the combined deficiencies, Bettger et al. (1979), designed a 35 day experiment and was able to show a zinc X linoleic acid interaction in weight gain and in epidermal arachidonic acid concentration within a time period that would not normally result in EFA deficiency signs. Clarke, Romsos, and Leveille (1977) investigated the time sequence of changes in hepatic fatty acid synthesis in rats meal-fed polyunsaturated fatty acids (PUFA) and demonstrated that the rate of fatty acid synthesis is decreased when rats are meal-fed PUFA for 3 days after being deprived of PUFA for 7 days. Therefore, both zinc and linoleic acid deficiencies can alter enzyme activity after relatively short periods of deprivation. Cunnane et al., (1984) have used an experimental period of 42 days to develop a zinc X linoleic acid interaction and have indicated that the 21 days used by Kramer et al., (1984) is insufficient to develop the full effects of zinc on EFA metabolism (Cunnane & Horrobin, 1985).

Experimental Parameters and Criteria

The purpose of this study was to investigate the interaction of dietary zinc and linoleic acid in the Sprague-Dawley rat. The parameters used to assess the interaction(s) were divided into two

categories. One category included parameters that could be measured while the experiment was in progress. These parameters which included food intake; weight gain; leukocyte alkaline phosphatase; bleeding time, and the total number of circulating leukocytes, granulocytes, and platelets in the animals fed the experimental diets were subjected to repeated measures analysis of variance. The second category included parameters measured at the end of the experiment. These parameters were subjected to univariate and multivariate analysis of variance and included appearance/muscle tone; dermal lesions; plasma zinc; plasma fatty acid profile, and testicular mineral concentrations of zinc, copper, iron, and manganese.

Previous investigations of dietary zinc and linoleic acid interactions in the laboratory rat have used natural lipid sources which contain many substances that can interfere with the experimental treatment and univariate statistics which do not consider the overall effect of the dietary treatment. The investigation described herein has eliminated these experimental problems by using purified linoleic acid and multivariate statistical techniques to access the overall effect of the dietary treatments.

CHAPTER III

EXPERIMENTAL METHODS

Design

A randomized block design, based on initial body weights, involving a 2^2 factorial treatment arrangement was used in this study. Factors included two levels of zinc (1.5 ppm and 40 ppm) and two levels of linoleic acid (0% and 2% of total dietary calories). Repeated measurements of the criteria evaluated were incorporated into the experiment, and the experimental phase consisted of 4 test diets which were fed to 24 rats for 33 days.

Diet Composition

The basic ingredients of the experimental diets used in this study consisted of carbohydrate[1], 73.0%; protein[2], 14.6%; palmitic acid[3], (3.5% or 4.0%); linoleic acid[4], (0.0% or 0.9%); vitamins[5], (1.0%);

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[1]Dextrose monohydrate, Teklad Test Diets, Madison, Wisconsin.

[2]Dried egg white solids (80% protein), Teklad Test Diets, Madison, Wisconsin.

[3]Mallinckrodt Incorporated, Paris, Kentucky.

[4]Fisher Scientific, Raleigh, North Carolina.

[5]AIN-76 vitamin mixture, Teklad Test Diets, Madison, Wisconsin.

minerals[6], (5%), and nonnutritive fiber[7] (2%). Percentages of the total caloric content of the diets provided by carbohydrate, protein, palmitic acid, and linoleic acid were 75%, 15%, 8% or 10%, and 2% or 0%, respectively. Analysis of the diets revealed that the zinc supplemented diets contained 40 ppm zinc versus 1.5 ppm zinc in the non-supplemented diets. The linoleic acid supplemented diets contained 85% palmitic acid, 6% stearic acid and 9% linoleic acid versus 93% palmitic acid and 7% stearic acid in the non-supplemented diets. The palmitic acid stock was contaminated with stearic acid. Approximately 150 mls of distilled/deionized water were added per kg of diet in order to reduce the spillage associated with powdered diets. Four diets were developed as follows: deficient zinc, deficient linoleic acid (ZDLD); adequate zinc, deficient linoleic acid (ZALD); deficient zinc, adequate linoleic acid (ZDLA); adequate zinc, adequate linoleic acid (ZALA). The composition of all diets, mineral mixture, and vitamin mixture are given in Appendix A.

Palmitic acid was used as the base fat source because it is a principle dietary fatty acid and its concentration in various tissues is apparently not sensitive to a fatty acid deficiency (Clejan et al., 1982; Huang et al., 1982; Cunnane, Horrobin, & Manku, 1984). Since water deprivation has been reported to enhance the effects of a fatty acid deficient diet (Holman, 1971), water was not restricted in this

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[6]Wesson Modified Osborne-Mendel mineral mixture, Teklad Test Diets, Madison, Wisconsin.

[7]Alphacel, ICN Nutritional Biochemicals, Cleveland, Ohio.

study in order to decrease the possibility of loss of experimental animals due to the combined stress of dietary and water deficiencies.

Animals

A total of 24 male albino Sprague-Dawley rats[8], approximately one month old, ranging in weight between 86 and 108 grams (mean 98 g), were used for the study. Upon receipt the animals were weighed, housed in suspended metabolic stainless steel cages, and fed the ZDLD diet for a 4 days preliminary period. Throughout the experiment all animals had free access to distilled/deionized water. After the preliminary period the animals were weighed and were randomly assigned to an experimental replication by body weight. The experimental diets were then assigned in a randomized block fashion to individual animals. All animals survived until the end of the experiment.

Protocol

Prior to receiving animals all equipment to be used in the experiment was washed and rinsed with ethylenediaminetetraacetic acid (EDTA). Water jar tops were wrapped in parafilm, and a dust cover was fitted around the animal cages. Glassware that was to be used in analytic procedures at the end of the experiment was acid washed. Throughout the experimental period cages and equipment were washed daily in an attempt to reduce environmental zinc contamination. Experimental diets containing linoleic acid were kept refrigerated and were made fresh every two weeks.

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[8]Holtzman Company, Madison, Wisconsin.

On experimental day 1 the animals were weighed, inspected, and placed on the experimental diets. The paws and muscle tone of each animal were evaluated, and a section of the left hind quarter was shaved on each rat. Since zinc deficiency is associated with hypophagia (Clejan, et al., 1982), food intake was recorded daily, and all animals were pair-fed an amount of food equal to the mean food intake of the ZDLD rats on the previous day. If the ZDLD rats consumed all of their diet, the amount of food offered on the following day was increased by 3 grams. In order to equalize food consumption among the experimental groups, food intake differences were adjusted between experimental days 15 and 20 so that all animals in all treatments would have equal caloric intakes. This adjustment was accomplished by reducing the amount of food offered to the animals fed the ZALD and ZALA diets. As a result of this adjustment the effect of diet and food intake on growth were confounded in animals fed the zinc adequate diets. The ambiguity associated with diet and food intake can be eliminated by assuming that food intake must be adjusted periodically to insure isocaloric consumption for all diets. Subsequent visual inspections and body weight determinations took place every 4th experimental day. The grading systems used to evaluate the appearance/muscle tone and paws of the experimental animals is given in Tables 1 and 2, respectively.

On experimental day 2 a bleeding time test was conducted. The hind quarter of the animal which had been shaved the previous day was washed with soap and water and allowed to dry. A sterile lancet was

used to prick the hind quarter, and a stop watch was used to record the bleeding time. Whatman number 1 filter paper was used to blot excess blood. When fresh blood no longer stained the filter paper, the watch was stopped, and the bleeding time was recorded. Subsequent bleeding time determinations were made on experimental days 12, 22, and 32.

Table 1

Grading System Used to Evaluate the Appearance/Muscle Tone of the Experimental Animals.

Rating	Criteria
1.	Appearance normal; firm body tonus, dandruff absent, no hair loss, tight fitting skin.
2.	Appearance deteriorating; soft body tonus, slight dandruff and hair loss, and loose fitting skin.
3.	Appearance dishevelled; flaccid body tonus, dandruff present, losing hair, and excess skin.

Table 2

Grading System Used to Evaluate the Front and Rear Paws of the Experimental Animals.

Rating	Criteria
1	Normal; no signs of scaling, glossing, erythema, cracking, or crusting.
2.	Slight scaliness.
3.	Definite scaliness, slight erythema and glossing.
4.	Definite scaliness, erythema and glossing,slight cracking.
5.	Definite scaling, erythema, glossing, cracking, and slight crusting.

On experimental day 3 approximately five units of sodium heparin[9] were injected into the base of the rat's tail. The animal was allowed to rest for 15 minutes. Blood was obtained by tail snip, using sharp scissors, for automated total leukocyte differential and platelet counts, and for a microscopic histochemical blood film count of leukocyte alkaline phosphatase activity. The first drop of blood to drip from the animals tail was blotted with filter paper. The second drop of blood was collected on a microscope slide and used for the leukocyte alkaline phosphatase activity determination. Subsequent drops of blood were collected in a test tube containing the anticoagulant EDTA. The blood specimens were submitted to Humana Hospital, Greensboro, N.C. for analysis. Prior to analysis the specimens were checked for clots. If a clot was found or if the sample could not be read, the specimen was discarded, and one degree of freedom was lost from the statistical analysis. Missing values were not estimated for statistical purposes. The total number of leukocytes, granulocytes, and platelets and the mean platelet volumes were determined using an S-PLUS Coulter Counter[10]. The total volume of blood intentionally removed from each animal per tail snip was limited to approximately 0.5 ml per 100 grams of body weight (Mitruka & Rawnsley, 1981). Subsequent blood sample collections by tail snip were taken on experimental days 13, 23, and 33.

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[9]Elkin-Sinn, Inc. Cherry Hill, New Jersey.

[10]Coulter Counter, S-PLUS; Humana Hospital, Greensboro, North Carolina.

Leukocyte alkaline phosphatase is found primarily in band and segmented PMN cells and the activity of the enzyme was demonstrated by simultaneously staining blood films with naphthol and diazonium salts which were purchased from Sigma Diagnostics[11]. Instructions (Appendix B, Table B-1) for the histochemical semiquantitative demonstration of leukocyte alkaline phosphatase activity in leukocytes were followed and the method of scoring is given in Table 3. The total score was the sum of the ratings of 33 PMN cells.

Table 3

Leukocyte Alkaline Phosphatase Activity Score

Rating	Amount[1]	Granule Size	Stain Intensity
0	None	---	None
1+	0-50	Small	Faint
2+	50-80	Small	Moderate
3+	80-100	Medium to Large	Strong
4+	100	Medium to Large	Brilliant

[1] Percentage of volume of cytoplasm occupied by azo dye precipitate.

The food was removed from all cages on experimental day 33, and on day 34 the animals were anesthetized with diethyl ether. Blood was collected, from each animal, in a beaker containing .5 ml heparin

[11] Sigma Diagnostics, St.Louis, Missouri.

(1000u/ml), by decapitation. The blood was diluted 1:1 with phosphate buffer (pH 7.4), mixed, and plasma was separated from cells by centrifugation. A 1 ml aliquot of plasma was then diluted in 1 ml of 6% trichloroacetic acid and .5 ml of saline. The resulting 1:5 dilution of plasma was centrifuged and the zinc concentration was determined by atomic absorption spectrophotometry[12] (AAS). Six standard zinc solutions (0.0, 0.05, 0.1, 0.2, 0.3, and 0.4 ppm zinc) were used to calibrate the AAS.

Lipids in a .5 ml aliquot of diluted plasma were extracted, cholesterol was removed, and the fatty acids were methylated using a procedure (Appendix B, Table B-2) obtained from the Department of Biochemistry, Bowman-Gray School of Medicine[13]. The methylated fatty acids were submitted to Bowman-Gray for analysis and were separated using gas-liquid chromatography (GLC)[14] equipped with a flame ionization detector and microprocessor to calculate the peak areas. An OV 351, 30 M X 0.25 mM fused silica capillary column[15], temperature programmed from 165°C, with an initial hold of 4 minutes, to 225°C at 3°C/minute, and a final hold of 13 minutes was used to separate the fatty acid methyl esters (FAMEs). The column flow was 0.68 ml/minute of helium as carrier gas and the split ratio was 1:99. Identification of individual fatty acids was made by comparison of the GLC output with

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[12]Model Video 12 E, Thermo Jarrell Ash Corp., Waltham, Massachusetts.

[13]Winston-Salem, North Carolina.

[14]Varian Model 3700, Varian Corporation, Houston, Texas.

[15]J + W Scientific, Rancho Cordova, California.

authentic methyl ester standards (Appendix B, Table B-3). The addition of a seventeen carbon saturated fatty acid internal standard (17:0[16]) made it possible to calculate the parts per million (ppm) of each fatty acid present in the sample.

The testes were removed, weighed, and ashed with nitric and perchloric acids on a hot plate. The ash of each pair of testes was dissolved in 3 ml of 0.6 N HCl and diluted to 25 mls with deionized water. The zinc, iron, calcium, and manganese concentration of the testes was determined by AAS.

Statistical Analysis

Data collected throughout the experimental phase were analyzed[17] using fixed effects repeated measures (RMANOVA), multivariate (MANOVA), or univariate (ANOVA) analysis of variance models. Statistical results were considered significant if the probability of observing such a result under the null hypothesis was less than 1 chance in 20. When either RMANOVA or MANOVA was employed, Roy's Maximum Root Criterion was used to test the hypothesis that the diets had no overall effect on the observed results.

In general, Roy's Maximum Root Criterion will consider all the variables in a model simultaneously and will reject the hypothesis of no overall systematic effect if at least one of the univariate tests conducted as part of the statistical procedure provides evidence that

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[16]Nu-Chek-Prep, Inc., Elysian, Minnesota.

[17]General Linear Models Procedure. SAS Institute Inc., Cary, North Carolina.

the diets have significantly influenced the results (Srivastava & Khatri, 1979). However, the null hypothesis of no difference among group mean vectors that is tested in the RMANOVA and MANOVA procedures depends on the joint distribution of all the dependent variables in the model and it is possible that the univariate results may not agree with the multivariate results. A Bonferroni 95% confidence interval for mean differences was computed for each significant univariate result.

RMANOVA has been used to answer two general questions in this investigation. The first question asks if there was any systematic change in the dependent variable group mean vectors over time. In order to understand the nature of changes over time a least squares quadratic regression equation was developed to express each dependent variable, for each animal, in terms of time. The intercepts, linear and quadratic coefficients of the individual regression lines were then subjected to MANOVA. The MANOVA results were used to conceptualize the changes in the dependent variable group mean vectors in terms of the linear and quadratic dimensions of time. The second question addressed by RMANOVA asks if the response of the dependent variables differed depending upon both the diet and the point in time, during the experimental phase, in which data was collected. MANOVA was also used to determine if the response variables differed depending upon the assigned level of the diet.

The assumptions of analysis of variance were accepted based on the following considerations (Neter, Wasserman, & Kutner, 1985): Provided that non-normality of the error distribution is not extreme, the F-test

is robust, and the mean contrast estimates are unbiased. In order to test the assumption of error term normality, a coefficient of correlation which relates the residuals to their expected values under the assumption of normality was computed for each variable and all error distributions were judged normal. When factor level sample sizes are approximately equal, the F-test for the equality of means is only slightly affected by unequal error variances. In order to test the assumption of equal error variances the Hartley test statistic (α = .01) was computed for each variable, and all error variances were judged approximately equal. Data sampling periods were selected to reduce carry-over effects (thus maximizing error term independence) while allowing for an evaluation of the quadratic effects of the treatments over time. Missing values were not estimated for statistical purposes. Sample sizes were predetermined by the number of stainless steel cages available for housing the experimental animals. In addition, the assumption that food intake must be adjusted periodically to insure isocaloric consumption for all diets has also been made.

CHAPTER IV

EXPERIMENTAL RESULTS

The raw data collected during the experimental phase of this research is given in Appendix C, and the statistical analyses of the raw data are given in Appendices D through F. Since differences among the experimental diet groups must be demonstrated before the results of this investigation can be used to support or refute the hypothesis of Bettger et al., (1979), the plasma levels of zinc and linoleic acid were examined to determine if the diets did significantly affect the characteristics measured in this research. The parameters that related to the hypothesis being tested were examined next. Finally the results relevant to the search for other dietary zinc and linoleic acid interactions were reviewed.

Results Relevant to the Experimental Diets

Plasma Zinc

Figure 2 illustrates the plasma zinc concentrations that were subject to ANOVA. Table D-1 indicates that the plasma zinc level was affected by dietary zinc ($p < .01$) and linoleic acid ($p < .03$) but not by a zinc X linoleic acid interaction. A test of the mean differences using the Bonferroni procedure indicated that rats consuming the ZA (ZALD and ZALA) diets had plasma zinc levels 34 (± standard deviation of 28) ppm higher than those consuming the ZD (ZDLD and ZDLA) diets.

Diets		Frequency	Mean
			ppm
ZDLD	**********	5	147
ZALD	**********	6	170
ZDLA	**********	4	103
ZALA	**********	6	152

50 100 150 200

Mean plasma zinc concentration

Figure 2. Bar Chart of Mean Plasma Zinc Concentrations.

A test of the mean difference associated with linoleic acid indicated that animals consuming the LD (ZALD and ZDLD) diets had plasma zinc levels approximately 27 (± 27) ppm higher than the animals consuming the LA (ZDLA and ZALA) diets, however, the 95% confidence interval included zero. Including zero in a mean difference confidence interval implies that a real difference between the means may not exist. Results of ANOVA and Bonferonni analysis can differ somewhat because error term variance may be reduced by including more independent variables in the ANOVA model.

Plasma Fatty Acids

Figure 3 illustrates the plasma fatty acid means which were subjected to MANOVA. Results of Roy's Maximum Root Criterion (Table E-1) indicated that linoleic acid had an overall significant effect ($p < .001$) on the plasma fatty acid profile. Table E-1 and the results of the Bonferroni tests also indicate that plasma linoleic acid concentrations were higher in the

Fatty Acid	Diet		Frequency	Mean
				ppm
Myristic	ZDLD	**	5	3.78
	ZALD	*	6	2.25
	ZDLA	*	4	2.49
	ZALA	*	6	2.19
Palmitic	ZDLD	**********	5	55.69
	ZALD	**********	6	49.69
	ZDLA	**********	4	67.23
	ZALA	**********	6	56.62
Stearic	ZDLD	**********	5	19.02
	ZALD	**********	6	19.14
	ZDLA	**********	4	21.94
	ZALA	**********	6	20.06
Oleic	ZDLD	**********	5	42.52
	ZALD	**********	6	37.02
	ZDLA	**********	4	44.27
	ZALA	**********	6	33.51
Linoleic	ZDLD	*	5	2.48
	ZALD	*	6	2.27
	ZDLA	**********	4	20.82
	ZALA	*******	6	14.72
Eicosa-trienoic	ZDLD	*	5	2.12
	ZALD		6	0.82
	ZDLA	*	4	1.58
	ZALA	*	4	1.63
Arachi-donic	ZDLD	*****	5	10.95
	ZALD	*****	6	9.11
	ZDLA	**********	4	35.56
	ZALA	**********	5	34.33

10 20 30 40 50 60

Mean Plasma Fatty Acid Concentration

Figure 3. Bar Chart of Plasma Fatty Acid Means.

animals fed the LA (ZDLA and ZALA) diets as compared to those fed the LD (ZDLD and ZALD) diets ($p < .001$). The Bonferroni results indicate that animals fed the LA diets had higher plasma linoleic acid (by 18 ± 4 ppm) concentrations than animals fed the LD diets. Results of this analysis also indicate that within this experimental design palmitic acid ($p < .05$) and arachidonic acid ($p < .001$) concentrations responded to dietary linoleic acid. Consumption of the LA diets resulted in a 10 (± 9) ppm higher plasma palmitic acid level and a 28 (± 7) ppm higher arachidonic acid level when compared to LD diet consumption.

Table D-2 lists the ANOVA results for the plasma ratio of eicosatrienoic acid to arachidonic acid (trienoic:tetraenoic ratio). Dietary zinc ($p < .04$), linoleic acid ($p < .003$), and the zinc X linoleic acid interaction ($p < .05$) had an effect on this ratio. Rats fed the ZD diets had a ratio .11 (± .1) points higher than rats fed the ZA diets, and rats consuming the LD diets had a ratio .15 (± .1) points higher than those fed the LA diets. Investigation of this interaction revealed that when linoleic acid was present in the diet the average ratio values were low (ZDLA ratio = .04 ± .01 and ZALA ratio = .04 ± .01), and when the diet was deficient in linoleic acid the average ratio values were higher (ZDLD ratio = .3 ± .17 and ZALD ratio = .11 ± .07). The highest ratio values were associated with the ZDLD diet which suggest that the rats fed the ZDLD diets were the most deficient in essential fatty acids. These results confirm the observation

of Bettger et al. (1979) that rats exhibit an exaggerated response to a combined nutrient deficiency since only the animals consuming the ZDLD diets came close to satisfying the Holman (1960) criteria for EFA deficiency based on the trienoic:tetraenoic ratio. These results also support the observation that deficiency signs can develop when ratio values are less than .4 (Huang et al., 1982).

Results Relevant to Growth

Weight Change

Figure 4 is a bar chart which lists the mean weight changes per diet over every 4 experimental days. Roy's Maximum Root Criterion confirms the decrease in weight change over time ($p < .001$) that is suggested in Figure 4 and identifies an overall time X zinc interaction ($p < .001$). The time X zinc interaction indicates that the pattern of weight change for the ZD and ZA diets were not the same across time. ANOVA results in Table F-1 indicate that dietary zinc had a significant effect on weight change for experimental days 2-5 ($p < .001$), 18-21 ($p < .001$), and 22-25 ($p < .02$). An interaction between zinc and linoleic acid ($p < .04$) was significant between experimental days 26 and 29. Investigation of this interaction revealed that the ZALA (2.6 g), ZDLA (.6 g), and ZDLD (5 g) diets were associated with an increase in weight for experimental days 26-29 but with the ZALD (-3 g) diet, weight decreased. That this interaction occurs at experimental days 26-29 suggests the effects of linoleic acid deficiency require time to develop and the weight loss associated

Days	Diet			Frequency	Mean
					g
2-5	ZDLD	|	**********	6	13.2
	ZALD	|	**********	6	23.5
	ZDLA	|	**********	6	9.5
	ZALA	|	**********	6	26.5
6-9	ZDLD	|	*****	6	5.2
	ZALD	|	**********	6	10.0
	ZDLA	|	*	6	0.6
	ZALA	|	*******	6	6.5
10-13	ZDLD	|	*	6	0.5
	ZALD	|	**********	6	9.6
	ZDLA	|	*********	6	8.6
	ZALA	|	**********	6	10.0
14-17	ZDLD	|	*******	6	7.3
	ZALD	|	******	6	6.3
	ZDLA	|	**	6	2.3
	ZALA	|	*********	6	8.6
18-21	ZDLD	|	*	6	0.6
	ZALD	********* |		6	-9.0
	ZDLA	|	*	6	1.0
	ZALA	******* |		6	-6.8
22-25	ZDLD	|	****	6	3.5
	ZALD	|	********	6	7.6
	ZDLA	|	****	6	3.6
	ZALA	|	*********	6	8.6
26-29	ZDLD	|	*****	6	5.0
	ZALD	*** |		6	-3.0
	ZDLA	|	*	6	0.6
	ZALA	|	***	6	2.6
30-33	ZDLD	* |		6	-0.8
	ZALD	* |		6	-0.6
	ZDLA	|	*	6	0.5
	ZALA	* |		6	-1.2

-5 0 5 10 15 20 25

Mean Weight Change

Figure 4. Bar Chart of Mean Weight Changes.

with linoleic acid deficiency can not be prevented by supplementing the diet with zinc. These results also suggest that a simultaneous deficiency of zinc may ameliorate the effects of linoleic acid deficiency. The adjustment in food consumption between days 15 and 20 could account, in part, for this interaction. In general, Figure 4 suggests that in this investigation the animals which were progressively deprived of a zinc adequate diet approached a zero weight change at about the same rate as animals which were consuming a zinc deficient diet.

The average change in weight per unit of time was computed (Table E-2) for each animal using quadratic regression analysis and the regression coefficients were subjected to MANOVA. Results of this analysis confirm that the changes in weight approached zero after approximately 24 days, as suggested in Figure 4, and that zinc contributed to both the linear effect over time ($p < .001$) and the quadratic effect over time ($p < .001$). Roy's Maximum Root Criterion confirmed that zinc had an overall effect on weight change ($p < .001$) but linoleic acid and the zinc X linoleic acid interaction did not affect weight change. Analysis of the linear and quadratic effects indicate that weight change in the animals consuming the ZA diets (Equation 1; ZA (Weight Change g) = $2.3 + (-2.9 \text{ time}) + (.74 \text{ time}^2)$) initially decreased more quickly than the weight change of animals consuming the ZD diets (Equation 2; ZD (Weight Change g) = $2.9 + (-1.0 \text{ time}) + (.19 \text{ time}^2)$). However, after 21 days weight change in the animals fed

the ZA diets was more positive so that by the end of the experiment weight change was approximately equal in all groups.

Food Intake

Figure 5 is a bar chart which indicates the mean food intake per diet over every 4 experimental days. RMANOVA in Table F-2 indicates that dietary zinc had a significant effect on food intake at every measurement period ($p < .04$) except days 18-21 during which time food intake was adjusted to equalize consumption among treatment groups. Roy's Maximum Root Criterion confirmed the decrease in food intake over time ($p < .001$) that is suggested in Figure 5 and identifies an overall time X zinc interaction ($p < .001$). The presence of a time X zinc interaction implies that the pattern of food intake was not the same for the ZA and ZD diets across time. The adjustment in food consumption between days 15 and 20 could account for this interaction. In general, Figure 5 suggests that the mean food intake of animals which consumed the ZA diets (ZALD = 47 ± 6 g and ZALA = 47 ± 7g) was greater than the animals which consumed the ZD diets (ZDLD = 42 ± 5 g and ZDLA = 41 ± 3 g).

The average food intake per unit of time was computed (Table E-3) for each animal and the regression coefficients were subjected to MANOVA. Results of this analysis indicate that zinc had an immediate effect on food intake ($p < .004$) since the intercept of the ZA diets (Equation 3; ZA (Food Intake g) = 45 + (-1.95 time) + (.46 $time^2$)) was higher than the intercept of the

Days	Diets		Frequency	Mean
				g
2-5	ZDLD	**********	6	49.3
	ZALD	**********	6	58.0
	ZDIA	**********	6	36.8
	ZAIA	**********	6	58.2
6-9	ZDLD	**********	6	48.6
	ZALD	**********	6	52.3
	ZDIA	**********	6	46.8
	ZAIA	**********	6	54.3
10-13	ZDLD	**********	6	34.2
	ZALD	**********	6	45.0
	ZDIA	**********	6	38.0
	ZAIA	**********	6	45.0
14-17	ZDLD	**********	6	43.3
	ZALD	**********	6	47.2
	ZDIA	**********	6	41.3
	ZAIA	**********	6	46.5
18-21	ZDLD	**********	6	43.3
	ZALD	**********	6	42.3
	ZDIA	**********	6	43.6
	ZAIA	**********	6	42.3
22-25	ZDLD	**********	6	40.5
	ZALD	**********	6	43.8
	ZDIA	**********	6	41.2
	ZAIA	**********	6	44.0
26-29	ZDLD	**********	6	39.7
	ZALD	**********	6	46.6
	ZDIA	**********	6	39.3
	ZAIA	**********	6	46.6
30-33	ZDLD	**********	6	38.6
	ZALD	**********	6	40.5
	ZDIA	**********	6	38.6
	ZAIA	**********	6	40.6

10 20 30 40 50

Mean Food Intake

Figure 5. Bar Chart of Mean Food Intakes.

ZD diets (Equation 4; ZD (Food Intake g) = 41 + (-.67 time) + (.01 $time^2$)). Table E-3 indicates an interaction between zincand linoleic acid contributed to the linear effect of the diets over time ($p < .03$) and that the quadratic effect of the diets over time was affected by zinc ($p < .008$). Roy's Maximum Root Criterion confirmed that zinc had an overall effect ($p < .001$) on the shapes of the response curves.

Analysis of the linear results indicate that animals consuming the ZA diets (ZALA linear coefficient = -2 and ZALD linear coefficient = -1.9) decreased their food intake at a faster rate than animals consuming the ZD diets (ZDLD linear coefficient = -1.2 and ZDLA linear coefficient = -.15). Investigation of this interaction revealed that when zinc was adequate in the diet the slope of food intake over time was more negative than when zinc was deficient in the diet. Animals consuming the ZA diets were pair-fed an amount of food equal to that consumed by the animals receiving the ZDLD diet. Since animals fed the ZA diets consistently consumed all their food ration, food restriction resulted in a high negative slope for food intake by these animals because the ZDLD rats consumed less food over time. Animals consuming the ZD diets were basically fed ad libitum, and animals fed the ZDLD diet decreased their food intake at a faster rate than animals consuming the ZDLA diet. This interaction suggests that a double deficiency which includes zinc (ZDLD) will result in a more rapid decrease in food consumption over time than will a

single deficiency (ZDIA) which involves zinc. Analysis of the quadratic effects over time indicate that food deprived rats fed a ZA diet (quadratic coefficient = .46) will consume more of the food offered to them than will ad libitum fed rats offered an equivalent mass of a ZD (quadratic coefficient = .01) diet.

Food Efficiency Ratio

The food efficiency ratio (FER = weight change X 100/ food intake) was subjected to RMANOVA. The results of the FER analysis (Table F-3) mirror the weight change results (Table F-2) because food intake was relatively uniform throughout this experiment. Table F-3 indicates that dietary zinc had a significant effect on FER for experimental days 2-5 ($p < .004$), 18-21 ($p < .001$), and 22-25 ($p < .03$). An interaction between zinc and linoleic acid ($p < .03$) was significant for experimental days 26-29. Investigation of this interaction revealed the same pattern noted in the weight change data; ZALA (5.7), ZDLA (0.3), and ZDLD (13.0) diets were associated with a positive FER between experimental days 26-29 but the FER associated with the ZALD (-6.3) diet was negative. Roy's Maximum Root Criterion results (Tables F-3) are consistent with the results of the weight change analysis (Tables F-2).

Results Relevant to Appearance

Appearance/Muscle Tonus (AMT) and Dermal Lesions

Figure 6 is bar chart illustrating the mean score per diet over every 4 experimental days for appearance/muscle tonus (AMT) based on a gross examination of the rats using the criteria given

in Table 1. Figures 7 and 8 list similar data based on the criteria in Table 2 for front and rear paw dermal lesions on the experimental animals. The results listed in Figures 6-8 indicate that as the experiment progressed in time the effects of the dietary deficiencies became more acute. The AMT scores for each rat were totalled over the entire experimental period and tested. From Tables C-4, C-5, and C-6 it is apparent that dermal lesions and AMT scores were fairly constant for all groups until late into the experiment. Therefore, application of RMANOVA to the 9 time periods would violate the basic assumptions of analysis of variance. Only the later time periods were tested with analysis of variance. A MANOVA (Table E-4) was developed based on the results of checking the normality of the data distributions. Results of Roy's Maximum Root Criterion indicate that dietary zinc had an overall effect on this multivariate model ($p < .05$). Table E-4 also indicates that neither dietary zinc or linoleic acid had a significant effect on the AMT scores. However, both zinc ($p < .05$) and linoleic acid ($p < .04$) had a significant effect on dermal lesions between experimental days 26 and 33. The Bonferroni test results indicate that animals consuming the ZD (3.2 ± 3) and LD (3.7 ± 2.8) diets had dermal lesion scores higher than the animals consuming the ZA or the LA diets respectively.

Days	Diets		Frequency	Mean Score
2-5	ZDLD	**********	6	1.8
	ZALD	**********	6	1.3
	ZDLA	**********	6	1.5
	ZALA	**********	6	1.2
6-9	ZDLD	**********	6	1.5
	ZALD	**********	6	1.2
	ZDLA	**********	6	1.2
	ZALA	**********	6	1.0
10-13	ZDLD	**********	6	1.6
	ZALD	**********	6	1.5
	ZDLA	**********	6	1.8
	ZALA	**********	6	1.2
14-17	ZDLD	**********	6	1.5
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.5
	ZALA	**********	6	1.0
18-21	ZDLD	**********	6	2.0
	ZALD	**********	6	1.2
	ZDLA	**********	6	1.8
	ZALA	**********	6	1.3
22-25	ZDLD	**********	6	2.0
	ZALD	**********	6	1.5
	ZDLA	**********	6	1.5
	ZALA	**********	6	1.3
26-29	ZDLD	**********	6	2.0
	ZALD	**********	6	1.2
	ZDLA	**********	6	1.3
	ZALA	**********	6	1.3
30-33	ZDLD	**********	6	1.5
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.5
	ZALA	**********	6	1.0

0.3 0.6 0.9 1.2 1.5 1.8

Mean Appearance/Muscle Tone Scores

Figure 6. Bar Chart of Appearance/Muscle Tone Scores.

Days	Diets		Frequency	Mean Score
2-5	ZDLD	**********	6	1.0
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.0
	ZALA	**********	6	1.0
6-9	ZDLD	**********	6	1.0
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.0
	ZALA	**********	6	1.0
10-13	ZDLD	**********	6	1.0
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.0
	ZALA	**********	6	1.0
14-17	ZDLD	************	6	1.2
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.0
	ZALA	**********	6	1.0
18-21	ZDLD	************	6	1.2
	ZALD	**********	6	1.0
	ZDLA	************	6	1.2
	ZALA	**********	6	1.0
22-25	ZDLD	**********************	6	2.2
	ZALD	**********	6	1.0
	ZDLA	*************	6	1.3
	ZALA	**********	6	1.0
26-29	ZDLD	***************************	6	2.6
	ZALD	******************	6	1.8
	ZDLA	******************	6	1.8
	ZALA	**********	6	1.0
30-33	ZDLD	******************************	6	3.0
	ZALD	******************	6	1.8
	ZDLA	**********************	6	2.2
	ZALA	************	6	1.2

1 2 3

Mean Front Paw Dermal Lesion Score

Figure 7. Bar Chart of Front Paw Dermal Lesion Scores.

Days	Diets		Frequency	Mean Score
2-5	ZDLD	**********	6	1.3
	ZALD	**********	6	1.3
	ZDLA	**********	6	1.3
	ZALA	**********	6	1.3
6-9	ZDLD	**********	6	1.5
	ZALD	**********	6	1.5
	ZDLA	**********	6	1.2
	ZALA	**********	6	1.2
10-13	ZDLD	**********	6	1.5
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.2
	ZALA	**********	6	1.0
14-17	ZDLD	**********	6	1.8
	ZALD	**********	6	1.0
	ZDLA	**********	6	1.0
	ZALA	**********	6	1.0
18-21	ZDLD	**********	6	2.5
	ZALD	**********	6	1.2
	ZDLA	**********	6	1.2
	ZALA	**********	6	1.0
22-25	ZDLD	**********	6	2.8
	ZALD	**********	6	1.3
	ZDLA	**********	6	1.3
	ZALA	**********	6	1.0
26-29	ZDLD	**********	6	3.2
	ZALD	**********	6	1.8
	ZDLA	**********	6	2.0
	ZALA	**********	6	1.0
30-33	ZDLD	**********	6	3.5
	ZALD	**********	6	2.0
	ZDLA	**********	6	1.6
	ZALA	**********	6	1.3

1 2 3

Mean Rear Paw Dermal Lesion Score

Figure 8. Bar Chart of Rear Paw Dermal Lesion Scores.

Results Relevant to Reproduction

Testicular Minerals

Figure 9 identifies the mean testicular concentrations of the minerals which were subjected to MANOVA. Results of Roy's Maximum Root Criterion (Table E-5) indicate that zinc ($p < .002$), had an overall significant effect on this multivariate model.

Mineral	Diet		Frequency	Mean
				mcg/g dry wt.
Copper	ZDLD	***	6	15.2
	ZALD	**	6	10.3
	ZDLA	***	6	17.3
	ZALA	**	6	12.1
Iron	ZDLD	**********	6	153.9
	ZALD	**********	6	100.7
	ZDLA	**********	6	275.7
	ZALA	**********	6	108.9
Zinc	ZDLD	**********	6	139.3
	ZALD	**********	6	175.7
	ZDLA	**********	6	161.9
	ZALA	**********	6	171.7
Manganese	ZDLD	*	6	3.5
	ZALD	*	6	2.3
	ZDLA	**	6	5.3
	ZALA	*	6	2.4

30 60 90 120 150 180 210 240 270

Mean Testicular Mineral Concentration

Figure 9. Bar Chart of Mean Testicular Mineral Concentrations.

Table E-5 indicates that the testicular copper ($p < .001$), iron ($p < .05$) and manganese ($p < .001$) concentrations were higher in the ZD compared to the ZA rats. The testicular manganese concentration was higher in the LA ($p < .03$) compared to the LD rats. The Bonferroni results indicate that copper was 5 ppm (± 2.5 ppm), iron 110 ppm (± 110 ppm), and manganese 2 ppm (± .84 ppm) higher in the testicles of the rats consuming the ZD diets compared to the ZA diets. However, the confidence interval for the mean difference in iron concentrations included zero. Table E-5 also indicates that within this experimental design the testicular manganese concentration responded to a dietary zinc X linoleic acid interaction ($p < .05$). Investigation of this interaction revealed that when zinc was adequate in the diet the average manganese concentrations were low (ZALD = 2.33 ±.18 ppm and ZALA = 2.43 ± .28 ppm), and when the diet was deficient in zinc the average concentrations were higher (ZDLD = 3.48 ± 1.39 ppm and ZDLA = 5.27 ± 1.42 ppm). Figure 10 illustrates this interaction.

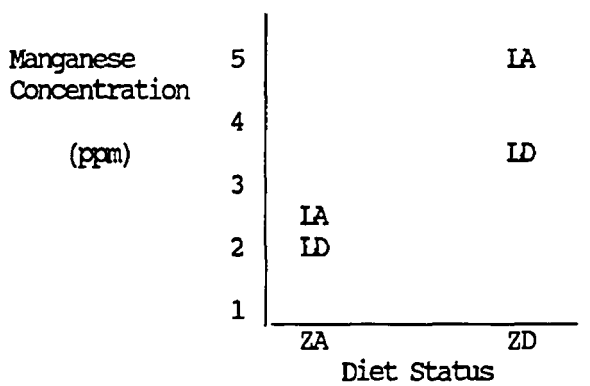

Figure 10. Interaction of zinc and linoleic acid on testicular manganese concentration.

The highest manganese concentrations were associated with the ZDLA diet which may suggest that in this experimental design LA made it possible to synthesize steroids at a normal rate and ZD made it necessary to substitute manganese for zinc.

Results Relevant to the Search For Additional Zinc and Linoleic Acid Interactions

Total Leukocyte Count (WBC)

Figure 11 is a bar chart which lists the mean total leukocyte counts per diet every 10 experimental days that were subjected to RMANOVA. Table F-4 indicates that dietary zinc had a significant effect on experimental day 23 ($p < .02$) and Roy's Maximum Root Criterion confirmed that the WBC decreased over time ($p < .001$) as suggested in Figure 11. The average decrease in WBC per unit of time was computed for each animal and the regression coefficients (Table E-6) were subjected to MANOVA. Results of this analysis indicate ZD (Equation 5; ZD (WBC X $10^3/mm^3$) = 11.5 + (-.88 time) + (.29 $time^2$)) had an immediate effect ($p < .005$) on the total leukocyte count since the intercept associated with the ZD diets was 3.39 (± 2.3) higher than the intercept for the ZA (Equation 6; WBC X $10^3/mm^3$) = 8.2 + (-1.53 time) + (1.39 $time^2$)) diets. Table E-6 also indicates that zinc contributed to the quadratic effect over time ($p < .03$) and that Roy's Maximum Root Criterion confirmed zinc had an overall effect ($p < .03$). Analysis of the effect over time indicates that animals consuming the ZA diets

Day	Diet		Frequency	Mean
				$10^3/mm^3$
3	ZDLD	**********	5	13.48
	ZALD	**********	5	13.96
	ZDLA	**********	5	14.48
	ZALA	**********	5	14.46
13	ZDLD	**********	5	11.66
	ZALD	**********	5	9.58
	ZDLA	**********	5	11.58
	ZALA	**********	5	10.32
23	ZDLD	**********	5	10.96
	ZALD	**********	5	7.44
	ZDLA	**********	5	10.44
	ZALA	**********	5	7.40
33	ZDLD	**********	5	11.26
	ZALD	**********	5	8.60
	ZDLA	**********	5	10.44
	ZALA	**********	5	9.68

2 4 6 8 10 12 14

Mean Total Leukocyte Counts

Figure 11. Bar Chart of Mean Total Leukocyte Counts

tended to have lower leukocyte counts compared to animals fed the ZD diets for the first 23 days of the experiment. After experimental day 23 leukocyte counts in animals consuming the ZA diets increased rapidly so by experimental day 33 the total leukocyte count was approximately equal for all treatment groups.

Absolute Granulocyte Count (PMN)

Figure 12 is a bar chart which lists the mean absolute granulocyte counts per diet every 10 experimental days that were subjected to RMANOVA. Table F-5 indicates that dietary zinc had a

Day	Diet		Frequency	Mean
				$10^3/mm^3$
3	ZDLD	**********	4	5.31
	ZALD	**********	5	4.83
	ZDLA	**********	4	6.15
	ZALA	**********	5	8.34
13	ZDLD	**********	4	4.51
	ZALD	**********	5	2.35
	ZDLA	**********	4	5.68
	ZALA	**********	5	2.43
23	ZDLD	**********	4	5.21
	ZALD	**********	5	1.74
	ZDLA	**********	4	4.33
	ZALA	**********	5	2.13
33	ZDLD	**********	4	1.42
	ZALD	**********	5	1.19
	ZDLA	**********	4	4.34
	ZALA	**********	5	2.15

1 2 3 4 5 6 7 8

Mean Absolute Granulocyte Count

Figure 12. Bar Chart of Mean Absolute Granulocyte Counts.

significant effect on the PMN on experimental days 13 ($p < .03$) and 23 ($p < .03$) and that Roy's Maximum Root Criterion confirmed that PMN decreased over time ($p < .001$) as suggested in Figure 12. The

average decrease in the PMN per unit of time was computed for each animal and the regression coefficients (Table E-7) were subjected to MANOVA. Results of this analysis indicate ZD (Equation 7; ZD (PMN X $10^3/mm^3$) = 4.5 + (-.16 time) + (.01 $time^2$) had an immediate effect ($p < .003$) on the absolute granulocyte count since the intercept associated with the ZD diets was 2.4 (± 1.5) higher than the intercept for the ZA (Equation 8; PMN X $10^3/mm^3$) = 2.1 + (-1.4 time) + (.8 $time^2$)) diets. Table E-7 also indicates that zinc contributed to a linear effect over time ($p < .03$) and that Roy's Maximum Root Criterion confirmed zinc had an overall effect ($p < .007$) on absolute granulocyte count. Analysis of the linear effect over time indicates that the PMN count was decreased in the animals consuming the ZA diets (slope = -1.4) at a faster rate than animals consuming the ZD diets (slope = -.16). Table F-5 indicates that by experimental day 33 the absolute granulocyte counts of the treatment groups were not significantly different.

Platelet Count (Plt)

Figure 13 is a bar chart which illustrates the mean platelet counts per diet every 10 experimental days that were subjected to RMANOVA. Table F-6 indicates that the platelet counts responded to a zinc X linoleic acid interaction on experimental day 3 ($p < .02$) but by experimental day 13 the interaction had dissolved into a main effect for zinc ($p < .06$) and a main effect for linoleic acid ($p < .05$). Investigation of the zinc X linoleic acid interaction that occurred on experimental day 3 revealed that when

Day	Diet		Frequency	Mean
				$10^3/mm^3$
3	ZDLD	**********	2	696
	ZALD	**********	5	1014
	ZDLA	**********	3	1357
	ZALA	**********	4	884
13	ZDLD	**********	2	1435
	ZALD	**********	5	1135
	ZDLA	**********	3	1116
	ZALA	**********	4	926
23	ZDLD	**********	2	847
	ZALD	**********	5	866
	ZDLA	**********	3	784
	ZALA	**********	4	820
33	ZDLD	**********	2	962
	ZALD	**********	5	944
	ZDLA	**********	3	626
	ZALA	**********	4	872

400 800 1200

Mean Platelet Counts

Figure 13. Bar Chart of Platelet Counts.

zinc was present in the diet the average ZA platelet counts (ZALD = 1015 and ZALA = 884) fell between the mean platelet counts associated with the ZD diets (ZDLD = 697 and ZDLA = 1357). This result suggests that a double deficiency (ZDLD) will depress the platelet count but a relative excess of linoleic acid (ZDLA), after a 7 day period of linoleic acid deprivation (4 days preliminary period plus 3 experimental days), will cause the number of platelets in the tip of a rats tail to increase. Figure 14 illustrates the zinc X linoleic acid interaction in platelet counts on experimental day 3.

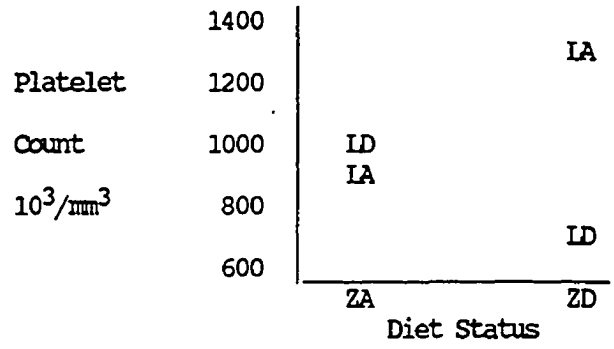

Figure 14. Interaction of Zinc and Linoleic Acid on Platelet Counts on Experimental Day 3.

Analysis of the marginally significant main effects on experimental day 13 indicate that the average platelet count of the animals fed the ZDLD diet had increased, which eliminated the zinc X linoleic acid interaction, and resulted in higher platelet counts in the ZD (mean = 1275) and LD (mean = 1285) diets compared to the ZA (mean = 1031) and LA (mean = 1021) diets.

Roy's Maximum Root Criterion (Table F-6) indicated that time ($p < .01$), time X linoleic acid ($p < .03$) and time X zinc X linoleic acid ($p < .06$) had an overall effect on the platelet count. In order to investigate the overall effect time had on platelet counts the average change in platelet count per unit of time was computed for each animal and the regression coefficients (Table E-8) were subjected to MANOVA. Results of this analysis indicate neither zinc or linoleic acid could account for the overall effect of time which suggests that the mean platelet counts change over time independent of zinc and linoleic acid.

Investigation of the time X linoleic acid interaction revealed that the platelet counts of rats fed the LA diets were initially higher than the platelet counts of the rats fed the LD diets. There was a progressive decrease in the platelet counts of LA rats over time but a tendency for the platelet counts of the LD rats to increase and then decrease. This result suggests that a greater number of platelets were required to stop bleeding in rats fed the LD diets. A marginally significant time X zinc X linoleic acid interaction (Figure 15) suggests that the pattern of platelet counts was not the same for all the diets across time. Figure 15 also suggests that the interpretation of the time X linoleic acid interaction could be extended to include both ZD and LD diets.

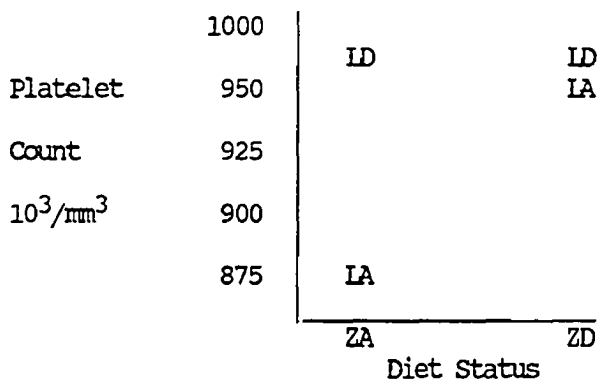

Figure 15. Interaction of Zinc and Linoleic Acid on Platelet Counts Averaged Over the Entire Study.

Apparently consuming either the ZD or the LD diets will increase the number of platelets required to stop bleeding caused by the

experimental treatments. In general these results suggest that both zinc and linoleic acid are necessary for optimum platelet function.

Mean Platelet Volume (MPV)

Figure 16 is a bar chart which illustrates the MPVs per diet every 10 experimental days that were subjected to RMANOVA. Table F-7 indicates that the MPV responded to a linoleic acid main effect on experimental days 23 ($p < .005$) and 33 ($p < .005$). Analysis of the linoleic acid effect using a Bonferroni 95% confidence interval revealed that rats fed the LD diets had larger platelet volumes on both experimental days 23 ($.41 \pm .25$) and 33 ($.34 \pm .24$) compared to the animals fed the LA diets.

Roy's Maximum Root Criterion (Table F-7) indicated that time ($p < .003$) had an overall effect on the MPV. This effect was investigated by calculating the average change in MPV per unit of time for each animal and subjecting the regression coefficients (Table E-9) to MANOVA. Results of this analysis indicate that linoleic acid had a significant effect on the linear changes in MPV ($p < .04$). Analysis of the linear result indicates that the MPV of rats fed the LD diets (Equation 9; LD (MPV) = 7 + (-.1 time) + (-.003 time^2)) decreased at a slower rate than the MPV of rats fed the LA diets (Equation 10; LA (MPV) = 6.9 + (-.22 time) + (-.029 time^2)). Further investigation revealed that the linear slope of the curve associated with the ZDLD diet (slope = -.03) was less negative than the linear slope of the ZALD diet

Day	Diet		Frequency	Mean
				fL
3	ZDLD	**********	2	7.01
	ZALD	**********	5	7.28
	ZDLA	**********	3	7.23
	ZALA	**********	4	7.23
13	ZDLD	**********	2	7.02
	ZALD	**********	5	7.06
	ZDLA	**********	3	6.77
	ZALA	**********	4	6.83
23	ZDLD	**********	2	7.00
	ZALD	**********	5	6.92
	ZDLA	**********	3	6.53
	ZALA	**********	4	6.53
33	ZDLD	**********	2	7.00
	ZALD	**********	5	6.70
	ZDLA	**********	3	6.37
	ZALA	**********	4	6.50

1 2 3 4 5 6 7

Mean Platelet Volume

Figure 16. Bar Chart of Mean Platelet Volumes.

(slope = -.17). Therefore, this result indicates that the MPV of rats fed the double deficiency diet (ZDLD) was more stable over time compared to the MPV of rats fed the other experimental diets.

Leukocyte Alkaline Phosphatase (LAP)

Figure 17 is a bar chart which illustrates the LAPs per diet every 10 experimental days that were subjected to RMANOVA. Table F-8 indicates that linoleic acid had an immediate main effect on

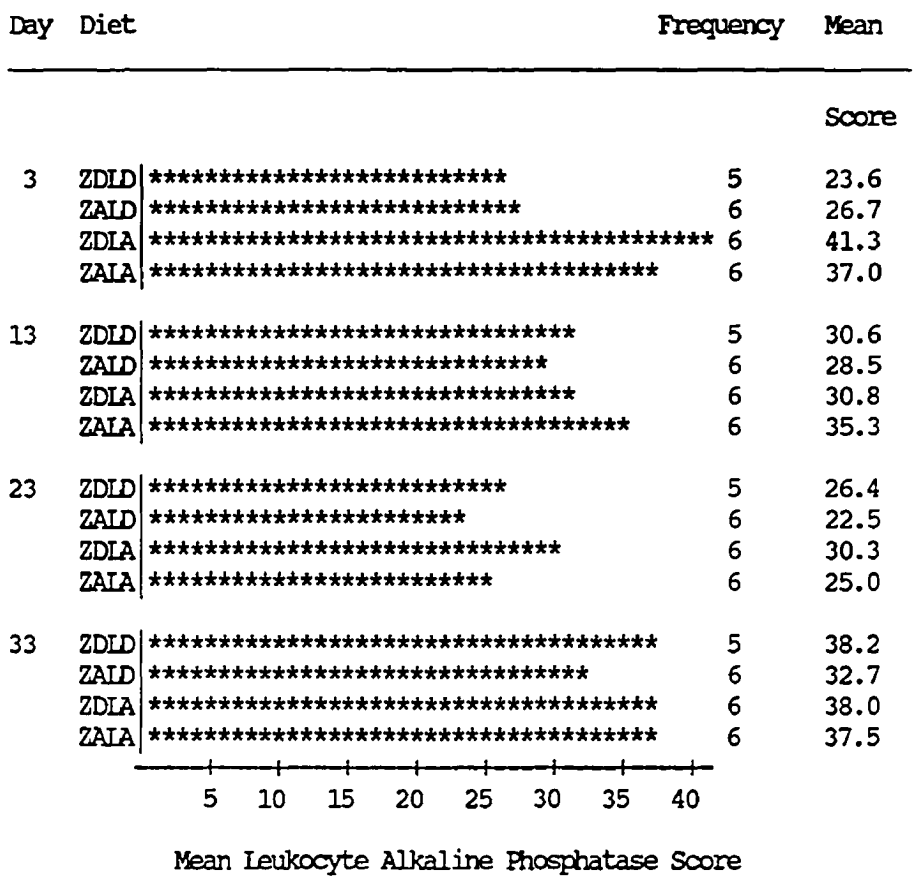

Figure 17. Bar Chart of Mean Leukocyte Alkaline Phosphatase Scores

LAP scores ($p < .003$), but this effect was not sustained. Analysis of the linoleic acid effect on experimental day 3 revealed that rats fed the LA diets had higher LAP score (13.9 ± 8.5) compared to the animals fed the LD diets.

Roy's Maximum Root Criterion (Table F-8) indicated that time ($p < .003$) had an overall effect on the LAP scores. The time effect was investigated by calculating the average change in LAP score per unit of time for each animal and subjecting the regression coefficients (Table E-10) to MANOVA. Results of this

analysis indicate that linoleic acid had a significant effect on the linear changes in IAP scores ($p < .03$). Analysis of the linear results indicate that the IAP scores of rats fed the LA diets (Equation 11; LA (IAP) = 29.4 + (-.97 time) + (4.0 $time^2$)) decreased slightly as the experiment progressed in contrast to an increase in IAP scores in the animals fed the LD diets (Equation 12; LD (IAP) = 26.3 + (3 time) + (2.73 $time^2$)).

Bleeding Time (BT)

Figure 18 is a bar chart which illustrates the BTs per diet every 10 experimental days that were subjected to RMANOVA.

Day	Diet		Frequency	Mean
				Seconds
2	ZDLD	**********	6	70
	ZALD	**********	6	71
	ZDLA	**********	6	56
	ZALA	**********	6	69
11	ZDLD	**********	6	41
	ZALD	**********	6	42
	ZDLA	**********	6	44
	ZALA	**********	6	51
22	ZDLD	**********	6	34
	ZALD	**********	6	40
	ZDLA	**********	6	54
	ZALA	**********	6	38
33	ZDLD	**********	6	27
	ZALD	**********	6	38
	ZDLA	**********	6	37
	ZALA	**********	6	28

0 10 20 30 40 50 60 70

Mean Bleeding Time

Figure 18. Bar Chart of Mean Bleeding Time

Table F-9 indicates that consuming the experimental diets did not result in a significant difference in BT. Roy's Maximum Root Criterion (Table F-9) indicated an overall time effect ($p < .001$) on the bleeding times. The time effect was investigated by calculating the average change in BT per unit of time for each animal and subjecting the regression coefficients (Table E-11) to MANOVA. Results of this analysis indicate neither dietary zinc or linoleic acid could account for the overall effect of time on the mean BT of animals in this investigation which suggest that the mean BTs changed over time independent of zinc and linoleic acid.

CHAPTER V

DISCUSSION

The purpose of this study was to investigate the interaction of dietary zinc and linoleic acid in the Sprague-Dawley rat, and to identify the approximate times during the experiment when interactions can be detected. Although rats were used as the experimental model, an understanding of the mechanisms underlying a zinc X linoleic acid interaction could have implications for the treatment of various diseases that plague man including: heart disease (Huang et al., 1982), acrodermatitis entropathica, diabetes, anorexia nervosa, and the glucagonoma syndrome (Horrobin et al., 1980). After Bettger et al. (1979) hypothesized that dietary zinc and linoleic acid interact metabolically, many researchers investigated the possibility that a decrease in the activity of delta-6 desaturase was the primary biochemical lesion responsible for growth retardation and teratogenesis associated with zinc deficiency (Dreosti et al., 1985). Horrobin et al. (1980) reviewed the evidence for an interaction between zinc and the essential fatty acids and concluded that substantial support for an interaction exists, but the nature of the interaction was not easy to understand. Fogerty, Ford, Dreosti, and Tinsley (1985) compared the results of several investigations which

demonstrated that zinc deficiency, reduced food intake, type of tissue (mammary, testis, or liver), type of experimental study (fatty acid composition or enzyme activity) and lipid fraction (phospholipid or neutral lipid) apparently influence delta-6 desaturase activity and concluded that the role of zinc in fatty acid metabolism was still an enigma. Since experiments which attempted to identify the most likely primary biochemical lesion associated with a zinc deficiency have yielded equivocal results, a general approach for identifing possible zinc X linoleic acid interactions patterns was undertaken in this investigation.

RMANOVA has been used to answer two general questions in this investigation. The first question asks if there were any systematic changes in the dependent variable group mean vectors over time. The results indicated that every dependent variable subjected to RMANOVA changed over time. In general, the time effect identified by RMANOVA was due to an overall effect of dietary zinc, although dietary linoleic acid did have a marginally significant effect on leukocyte alkaline phosphatase scores. Detecting a systematic main effect across time would imply that a fundamental relationship existed between a variable which has been manipulated and a parameter which has been measured.

In this investigation the consumption of a zinc adequate diet was associated with systematically increased food intake and weight gain, but decreased total leukocyte and absolute granulocyte counts. These main effects are well established in

the nutrition literature. The purpose of this study was to determine if zinc and linoleic acid systematically interact across time. Further analysis of the time effect revealed no overall patterns of zinc X linoleic acid interactions in the dependent variables subjected to RMANOVA. Detecting a systematic interaction over time would imply that food intake, weight change, and the total leukocyte and absolute granulocyte counts depended upon particular combinations of zinc and linoleic acid in the diet. The fact that interactions across time were not detected suggests that other dietary and/or physiological factors, in addition to the combinations of zinc and linoleic acid used in this experiment, control the systematic response of these parameters in the laboratory rat. The changes over time observed in platelet counts, mean platelet volumes, and in bleeding times were not associated with the dietary treatments and probably reflected normal physiological changes.

The second question addressed by RMANOVA asks if the responses of the dependent variables differed depending upon both the assigned level of diet and the point in time, during the experimental phase, in which data were collected. The results indicated that the group mean vectors of weight change, food intake, and FER were influenced by an overall time X zinc effect. Detecting significant differences, therefore, depended upon sampling at specific times during the experiment. Bettger et al. (1979) found a univariate zinc X EFA interaction in weight change

after 35 days. In this investigation a univariate zinc X linoleic acid interaction developed after 26-29 days (Table F-1). Therefore, in this investigation the Bettger et al., (1979) hypothesis that growth in rats is affected by a zinc X linoleic acid interaction was supported by univariate analyses, but the interaction was not strong enough to cause significant multivariate results. Results of the weight change analysis support the observation by Cunnane et al., (1984) that more than 21 days were required to develop the effects of zinc and essential fatty acid on the growth of rats. The results also indicated that a zinc X essential fatty acid interaction will develop in less than 42 days as suggested by Krammer et al. (1984).

In terms of the possible relationship of zinc to physiological function illustrated in Figure 1, the results associated with weight change and FER (as indicators of growth) suggest that possible times for detecting a zinc effect may be between experimental days 2-5, 18-21, and 22-25. These intervals support the observation that zinc exerts a cyclic effect on weight change and suggest that as the deficiency progresses in time the cycles lengthen. Analysis of the food intake results suggests rats fed the ZD diet will consume less food as an experiment progresses. When an experiment includes both ZA and ZD diets, food intake can only be kept equal if the rats consuming ZA diets are starved. Therefore, when the effects of consuming a ZD versus ZA diet are evaluated, the actual comparison is between

the effects of ZD and starvation. Neither zinc deficiency nor starvation had an easily identifible affect on the plasma fatty acid profile used in this investigation. Based on RMANOVA analysis the plasma fatty acid profile was altered primarily by dietary linoleic acid and only marginally altered by the zinc concentrations in the diet. Therefore, the results of this study imply that neither zinc deficiency, as suggested by Cunnane and Horrobin (1985) nor reduced food intake (starvation), as suggested by Krammer et al. (1984), has a strong influence on delta-6 desaturase which mediates the conversion of linoleic acid to arachidonic acid. These results leave open the possibility that zinc influences other desaturases that are involved in the conversion of linoleic acid to higher homologues.

A modification of the feeding procedure used by Clarke et al. (1977) was used in this study and resulted in the detection of significant zinc X linoleic acid interactions in platelet counts. Basically, the diet feeding protocol required that rats consume an essential nutrient deficient diet (ZDLD) for a few days followed by supplementation of the deficient nutrients (ZDLA, ZALD, and ZALA). The feeding procedure specifically recognizes the possibility that 'steady states' of metabolic activity can be established within days after an essential nutrient is withdrawn then introduced into a diet. Introducing the deficient nutrients into the diet after the animals had been fed a zinc deficient diet during the preliminary phase resulted in an overall time X

linoleic acid interaction and a time X zinc X linoleic acid interaction in the platelet count group mean vectors. In terms of Figure 1 the results suggest that the optimum time for detecting a linoleic acid effect on the platelet count may be around experimental day 13. The optimum time for detecting a zinc X linoleic acid effect on the platelet counts may be around experimental day 3 given the protocol followed in this study.

MANOVA was used to determine if the responses of the dependent variables differed depending upon the assigned level of zinc and linoleic acid. The results indicate that dermal lesion scores and testicular mineral concentrations, but not appearance/muscle tonus scores, were affected by the level of zinc consumed in the diet. The results also indicate that both zinc and linoleic acid exert main effects on paw dermal lesions in the rat. However, an interaction was not found in the dermal lesion scores, so the results of this investigation do not support the Bettger et al. (1979) hypothesis that dermal lesions in the rat are affected by a zinc X linoleic acid interaction. It is noted that in the original article by Bettger et al. (1979) the hypothesis of a zinc X essential fatty acid interaction on growth and in arachidonic acid metabolism was based on a statistical analysis of the data. However, the hypothesis that skin lesions respond to unique combinations of zinc and essential fatty acids in the diet was based on a subjective interpretation of the data. Results of this investigation suggest that within 33 days dermal

lesions in the rat do not develop as a result of a zinc X essential fatty acid interaction.

Dietary zinc exerted an overall effect on the testicular mineral profile. A marginally significant overall zinc X linoleic acid interaction was also detected. The univariate results indicate that both zinc and linoleic acid exert main effects on the concentration of testicular manganese. Results of this investigation indicated that consumption of the experimental diets for 33 days result in a zinc X linoleic acid interaction which will influence the concentration of manganese in the testes of the rat. This interaction supports the observation of Roth and Kirchgessner (1977) that short term zinc deficiency will cause the manganese concentration in some tissues to increase. These results also support the Bettger et al. (1979) hypothesis that zinc and linoleic acid interact in the reproductive organs of the rat.

MANOVA was also applied to the variables included in the plasma fatty acid profile. In addition to the overall effect of linoleic acid, zinc and the zinc X linoleic acid interaction had marginally significant overall effects on the fatty acid profile. An overall multivariate effect of zinc and linoleic acid on a plasma fatty acid profile has not been previously reported in the literature. These results suggest that the decision to use purified fatty acids rather than natural fat sources optimized

the chance of detecting zinc and linoleic acid interactions in the laboratory rat.

In general, the results of this investigation indicate, as Horrobin et al. (1980) have observed, that substantial support for a dietary zinc X linoleic acid interaction exists, but the nature of the interaction is not easy to understand. This investigation attempted to discern an over all pattern in zinc X linoleic acid interactions by observing the number, volume, concentration or appearance of selected physiological variables and characteristics in young rats fed diets with and without zinc and linoleic acid. Although consumption of the experimental diets did result in zinc X linoleic acid interaction in the laboratory rats, a clear pattern to the interactions did not emerge.

CHAPTER VI

SUMMARY AND RECOMMENDATIONS

The results of this study support the hypothesis that dietary zinc and linoleic acid interact in the laboratory rat. This investigation has identified growth, testicular manganese, platelet count, and the plasma fatty acid profile as physiological parameters that respond to a zinc X linoleic acid interaction. In addition, the time element associated with the onset of a dietary interaction in weight change and platelet count has been approximated. The results of this study suggest that the similarities of the skin lesion signs that develop when either zinc or essential fatty acids are deficient in the diet of a weanling rat do not necessarily imly that these nutrients interact metabolically. The results also suggest that zinc deficiency and reduced food intake (starvation) had comparable affects on the plasma fatty acid profile used in this study.

Future investigations should focus on developing diets that contain the maximum amount of zinc and linoleic acid which will reliably produce deficiency signs when consumed by rats, and on developing reliable non-traumatic methods for blood collection. More reliable diets and blood collection techniques may allow for a more comprehensive exploration of the mechanisms responsible for the nutrient interactions.

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APPENDIX A

COMPOSITION OF THE EXPERIMENTAL DIETS

Table A-1

Composition of Experimental Diets.

Constituent[a]	Diet[b]			
	ZDLD	ZDLA	ZALD	ZALA
	g/kg			
Dextrose monohydrate	730	730	730	730
Dried egg white solids	146	146	146	146
Palmitic acid	44	35	44	35
Linoleic acid	0	9	0	9
Mineral mix	50	50	50	50
Vitamin mix	10	10	10	10
Alphacell	20	20	20	20
Zinc sulfate	0	0	0.1[c]	0.1

[a]150 ml distilled/deionized water added per kg of diet.

[b]Diets were isocaloric at approximately 3.9 Kcal/g (calculated). Nomenclature for diet designation: zinc (Z), linoleic acid (L), adequate (A), and deficient (D).

[c]Yields 40 ppm Zinc.

Table A-2

Composition of Mineral Mix, Wesson Modified Osborne-Mendel[a]

Constituents	g/kg
Calcium carbonate	21.000
Copper sulfate-5 H_2O	.039
Ferric phosphate	1.470
Manganous sulfate (anhydrous)	0.020
Magnesium sulfate (anhydrous)	9.000
Potassium aluminum sulfate	0.009
Potassium chloride	12.000
Potassium dihydrogen phosphate	31.000
Potassium iodide	0.005
Sodium chloride	10.500
Sodium fluoride	0.057
Tricalcium phosphate	14.900

[a]Teklad Test Diets, Madison, Wisconsin.

Table A-3

Composition of Vitamin Mix, AIN[a]

Constituents	Amount
	g/kg
Thiamin HCL	0.6
Riboflavin	0.6
Pyridoxine HCL	0.7
Niacin	3.0
Calcium pantothenate	1.6
Folic acid	0.2
Biotin	0.02
Vitamin B_{12} (0.1% trituration in mannitol)	1.0
Vitamin A Palmitate (500,000 U/g)	0.8
Vitamin D_3 trituration (400,000 U/g)	0.25
Vitamin E acetate (500 U/g)	10.0
Menadione	0.005
Sucrose, finely powdered	981.225

[a]Teklad Test Diets, Madison, Wisconsin.

Appendix B

Analytic Procedures

and Composition of Standards

Table B-1

Procedure for the Preparation of Blood Films for the Histochemical Semiquantitative Demonstration of Alkaline Phosphatase in Leukocytes (Procedure 86-R[a]).

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1. Fix slides in citrate-acetone-formaldehyde solution for 30 seconds and rinse thoroughly in deionized water.

2. Adjust temperature of 45 ml deionized water to 23-27°C.

3. To 1 ml of FRV-Alkaline solution, add 1 ml sodium nitrate solution, and vortex vigorously for 30 seconds.

4. Add solution from step 2 to deionized water from step 3.

5. Add 1 ml naphthol AS-BI alkaline solution, mix thoroughly and pour into coplin jar.

6. Add slides and incubate, protected from light, at room temperature for 15 minutes.

7. After 15 minutes, remove slides and rinse thoroughly in deionized water.

8. Counterstain in hematoxylin solution, Gill # 3, for 2 minutes.

9. Rinse thoroughly and air dry.

10. Mount slides in water, coverslip and examine using oil immersion objective.

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[a]Reagents and analytic procedure were supplied by Sigma Diagnostics, St. Louis, Missouri.

Table B-2

Procedure[a] for the Preparation of Plasma Lipids Prior to Analysis by Gas-Liquid Chromatography.

Lipid Extraction

To a .5 ml sample of dilute plasma in a 15 ml centrifude tube (Teflon-lined, with screw cap):

add 1.0 ml methanol (HPLC grade), vortex,
add 0.5 ml chloroform (HPLC grade), mix,
add 0.5 ml 0.9% saline and 1.0 ml chloroform, mix.

Let sample stand at room temperature until 2 phases have separated, the bottom phase contains the neutral lipids.

Removal of Cholesterol

Dry known amount of lipid extract in a 15 ml centrifuge tube (Teflon-lined, with screw cap):

add 3 ml of 95% ethanol, while mixing add 0.3 ml of 33% potassium hydroxide, purge tube with nitrogen gas, cap tightly, place in 60°C heating block for 15 minutes,
cool tube, add 3 ml water and 10 mL hexane (HPLC grade), cap tube tightly,
shake tube vigorously for 30 seconds, allow tube to stand at room temperature until phases separate, remove upper phase and disgard, vortex lower phase, add 8 drops of concentrated sulfuric acid, add 10 ml hexane, cap tube, shake for 30 seconds, allow phases to separate.

Transfer top phase to clean screw cap tube, dry sample under nitrogen.

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[a]Procedure currently used by the Department of Biochemistry, Bowman-Gray School of Medicine, Winston-Salem, N.C.

Table B-2 (continued)

Procedure for the Preparation of Plasma Lipids Prior to Analysis by Gas-Liquid Chromatography.

Methylation

To the lipid extract:

add 50 lambda of 17:0 internal standard,
add 2.5 ml methanol, while vortexing add 0.2 ml concentrated sulfuric acid dropwise,
flush tube with nitrogen gas, seal, heat at 85°C for 2.5 hours, cool to room temperature,
add 2.5 ml pentane (HPLC grade) and 2.0 mL water, cap, shake vigorously for 30 seconds.

Transfer top lay to a clean test tube, dry under nitrogen gas, and resuspend in 1.0 ml undecane (HPLC grade). Submit samples for GLC analysis.

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[a]Nu-Chek-Prep, Inc. Elysian, Minnesota.

Table B-3

Gas Liquid Chromatography Reference Fatty Acid Standards[a] (Mixture 68B).

Fatty Acid Name	Chain Length	Location of Double Bonds	Percent by Weight
Methyl Myristate	14:0		3.0
Methyl Myristoleate	14:1	9	1.0
Methyl Palmitate	16:0		10.0
Methyl Palmitoleate	16:1	9	2.0
Methyl Stearate	18:0		15.0
Methyl Oleate	18:1	9	25.0
Methyl Linoleate	18:2	9,12	10.0
Methyl Linolenate	18:3	9,12,15	4.0
Methyl Arachidate	20:0		2.0
Methyl Eicosenoate	20:1	11	2.0
Methyl Eicosadienoate	20:2	11,14	2.0
Methyl Homogamma Linolenate	20:3	8,11,14	4.0
Methyl Arachidonate	20:4	5,8,11,14	4.0
Methyl Behenate	22:0		4.0
Methyl Erucate	22:1	13	2.0
Methyl Docosahexaenoate	22:6	4,7,10,13,16,19	4.0
Methyl Lignocerate	24:0		2.0
Methyl Nervonate	24:1	15	4.0

[a]Nu-Chek-Prep, Inc., Elysian, Minnesota.

Appendix C

Raw Data

Table C-1

Food Intake of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Total Food Intake For Experimental Days							
		2-5	6-9	10-13	14-17	18-21	22-25	26-29	30-33
					g				
1	ZDLD	44	39	37	45	35	41	32	34
6		67	50	29	51	44	41	46	42
10		58	54	35	37	46	45	47	39
15		41	48	37	47	45	45	45	39
17		43	47	32	38	41	30	33	38
21		43	54	35	42	49	41	35	40
3	ZALD	64	50	48	51	38	43	46	42
7		64	50	48	51	38	44	46	42
11		61	53	45	49	43	45	48	40
13		55	53	45	46	43	45	48	39
20		52	54	42	43	46	43	46	40
23		52	54	42	43	46	43	46	40
4	ZDLA	49	44	34	42	46	42	33	38
8		35	50	37	41	45	41	44	41
9		19	51	48	41	41	43	35	39
14		52	48	41	46	46	45	48	39
19		38	48	36	41	46	42	42	40
24		28	40	32	37	38	34	34	35
2	ZALA	67	50	48	51	38	44	46	42
5		67	50	48	51	38	44	46	42
12		58	53	45	46	43	45	48	41
16		54	53	45	45	43	45	48	39
18		51	59	42	43	46	43	46	40
22		52	61	42	43	46	43	46	40

Table C-2

Weight Change of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Total Weight Change For Experimental Days							
		2-5	6-9	10-13	14-17	18-21	22-25	26-39	30-33
		g							
1	ZDLD	8	10	8	2	6	2	3	0
6		20	0	5	15	-1	2	4	5
10		20	17	-5	9	1	8	1	1
15		10	6	-7	6	2	1	7	-5
17		8	0	-1	9	-2	1	4	-4
21		13	-2	3	3	-2	7	11	-2
3	ZALD	28	4	19	11	-13	10	-5	-3
7		27	3	26	5	-14	15	-6	2
11		20	12	4	4	-11	8	-8	-1
13		26	13	3	17	-9	7	-6	-3
20		12	16	3	-4	-5	5	6	-1
23		28	12	3	5	-2	1	1	2
4	ZDLA	25	-13	18	-1	0	11	-6	-3
8		11	8	10	3	5	6	-4	-6
9		-5	15	6	1	9	0	-2	9
14		13	2	8	-1	3	0	10	-1
19		6	-4	1	8	-4	3	7	7
24		7	-4	9	4	-7	2	-1	-3
2	ZALA	30	0	24	7	-10	10	-2	-3
5		30	0	20	8	-18	16	-7	-2
12		29	17	3	19	-4	12	4	4
16		22	12	2	18	-9	8	0	-2
18		19	7	5	1	-3	-1	11	-4
22		29	3	6	-1	3	7	10	0

Table C-3

Food Efficiency Ratio of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Food Efficiency Ratio For Experimental Days							
		2-5	6-9	10-13	14-17	18-21	22-25	26-29	30-33
		(g weight change/g food intake) * 100							
1	ZDLD	18.18	25.64	21.62	4.44	17.14	4.87	9.37	0.00
6		29.85	0.00	17.24	29.41	-2.27	4.87	8.69	11.90
10		34.48	31.48	-14.28	24.32	2.17	17.77	2.12	2.56
15		24.39	12.50	-18.91	12.76	4.44	2.22	15.55	-12.82
17		18.60	0.00	-3.12	23.68	-4.87	3.33	12.12	-10.52
21		30.23	-3.70	8.57	7.14	-4.08	17.07	31.42	-5.00
3	ZALD	43.75	8.00	39.58	21.56	-34.21	23.25	-10.87	-7.14
7		42.18	6.00	54.16	9.80	-36.84	34.09	-13.04	4.76
11		32.78	22.64	8.88	8.16	-25.58	17.77	-16.66	-2.50
13		47.27	24.52	6.66	36.95	-20.93	15.55	-12.50	-7.69
20		23.07	29.62	7.14	-9.30	-10.87	11.62	13.04	-2.50
23		53.84	22.22	7.14	11.62	-4.34	2.32	2.17	5.00
4	ZDLA	51.02	-29.54	52.94	-2.38	0.00	26.19	-18.18	-7.89
8		31.42	16.00	27.02	7.31	11.11	14.63	-9.09	-14.63
9		-26.31	29.41	12.50	2.43	21.95	0.00	-5.71	23.07
14		25.00	4.16	19.51	-2.17	6.52	0.00	20.83	-2.56
19		15.78	-8.33	2.77	19.51	-8.69	7.14	16.66	17.50
24		25.00	-10.00	28.12	10.81	-18.42	5.88	-2.94	-8.57
2	ZALA	44.77	0.00	50.00	13.72	-26.31	22.72	-4.34	-7.14
5		44.77	0.00	41.66	15.68	-47.36	36.36	-15.21	-4.76
12		50.00	32.07	6.66	41.30	-9.30	26.66	8.33	9.75
16		40.74	22.64	4.44	40.00	-20.93	17.77	0.00	-5.12
18		37.25	11.86	11.90	2.32	-6.52	-2.32	23.91	-10.00
22		55.76	4.91	14.28	-2.32	6.52	16.27	21.73	0.00

Table C-4

Front Paw Dermal Lesions on Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Front Paw Dermal Lesions On Experimental Day								
		1	5	9	13	17	21	25	29	33
		Score[a]								
1	ZDLD	1	1	1	1	1	1	1	2	2
6		1	1	1	1	1	1	3	3	3
10		1	1	1	1	1	1	1	3	3
15		1	1	1	1	1	1	3	4	5
17		1	1	1	1	1	1	3	3	5
21		1	1	1	1	2	2	2	3	4
3	ZALD	1	1	1	1	1	1	1	3	2
7		1	1	1	1	1	1	1	1	2
11		1	1	1	1	1	1	1	2	2
13		1	1	1	1	1	1	1	2	2
20		1	1	1	1	1	1	1	2	1
23		1	1	1	1	1	1	1	1	1
4	ZDLA	1	1	1	1	1	1	1	1	3
8		1	1	1	1	1	1	1	1	2
9		1	1	1	1	1	1	1	1	3
14		1	1	1	1	1	1	1	2	3
19		1	1	1	1	1	1	1	2	1
24		1	1	1	1	1	2	3	4	3
2	ZALA	1	1	1	1	1	1	1	1	1
5		1	1	1	1	1	1	1	1	1
12		1	1	1	1	1	1	1	1	1
16		1	1	1	1	1	1	1	1	2
18		1	1	1	1	1	1	1	1	1
22		1	1	1	1	1	1	1	1	1

[a] From Table 2, page 24.

Table C-5

Rear Paw Dermal Lesions On Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Rear Paw Dermal Lesions on Experimental Day								
		1	5	9	13	17	21	25	29	33
		Score[a]								
1	ZDLD	1	1	1	2	1	2	3	3	4
6		1	1	1	2	3	3	4	4	5
10		1	1	1	1	1	2	1	3	3
15		1	3	3	2	1	3	3	4	5
17		1	1	2	1	2	2	3	3	5
21		1	1	1	1	3	3	3	4	4
3	ZALD	1	2	1	1	1	2	2	3	3
7		1	1	1	1	1	1	1	1	2
11		1	2	2	1	1	1	1	2	2
13		1	1	2	1	1	1	2	2	4
20		1	1	1	1	1	1	2	1	3
23		1	2	2	1	1	1	1	1	1
4	ZDLA	1	1	1	2	1	1	1	2	2
8		1	1	1	1	1	1	1	2	1
9		1	2	2	1	1	1	1	1	1
14		1	2	1	1	1	1	1	2	3
19		1	1	1	1	1	1	1	1	1
24		1	1	1	1	1	2	3	4	3
2	ZALA	1	1	1	1	1	1	1	1	1
5		1	1	1	1	1	1	1	1	1
12		1	2	2	1	1	1	1	1	1
16		1	2	1	1	1	1	1	1	3
18		1	1	1	1	1	1	1	1	1
22		1	1	1	1	1	1	1	1	1

[a] From Table 2, page 24.

Table C-6

Appearance/Muscle Tone of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Muscle Tone On Experimental Day								
		1	5	9	13	17	21	25	29	33
		Score[a]								
1	ZDLD	1	2	1	2	2	2	2	2	2
6		1	2	2	1	3	1	3	2	1
10		1	2	1	1	1	2	1	2	1
15		1	2	2	3	1	2	2	2	2
17		1	2	2	3	2	3	2	3	2
21		1	1	1	1	1	2	2	2	1
3	ZALD	1	1	1	1	1	1	1	2	1
7		1	1	1	1	1	1	2	1	1
11		1	2	1	2	1	1	2	2	1
13		1	2	2	2	1	1	1	1	1
20		1	1	1	1	1	2	3	2	1
23		1	1	1	2	1	1	3	2	2
4	ZDLA	1	1	1	1	1	1	1	2	1
8		1	2	1	2	1	1	2	2	1
9		1	2	1	2	2	2	2	2	2
14		1	2	2	2	1	3	2	2	2
19		1	1	1	3	3	3	2	1	2
24		1	1	1	1	1	1	2	1	2
2	ZALA	1	1	1	1	1	1	2	1	1
5		1	1	1	1	1	1	1	1	1
12		1	1	1	1	1	1	1	1	1
16		1	2	1	2	1	2	1	2	1
18		1	1	1	1	1	2	2	1	1
22		1	1	1	1	1	1	2	2	1

[a] From Table 1, page 23.

Table C-7

Total Leukocyte and Absolute Granulocyte Count of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Total Leukocytes Experimental Day				Absolute Granulocytes Experimental Day			
		3	13	23	33	3	13	23	33
		Count ($10^3/mm^3$)				Count ($10^3/mm^3$)			
1	ZDLD	16.5	14.0	14.0	14.4	6.27	.42	6.16	2.30
6		11.6	11.6	8.0	9.7	2.55	5.45	1.52	.58
10		12.3	7.6	17.1	12.1	6.15	1.52	11.63	2.06
15		14.8	14.0	15.1	---[a]	6.66	2.38	6.79	---
17		10.5	11.1	8.8	10.9	1.68	---	---	4.45
21		16.5	14.0	6.9	9.2	6.27	6.86	1.52	.74
3	ZALD	20.6	16.7	9.6	13.3	3.94	4.68	.96	1.33
7		14.1	10.7	7.7	7.6	3.53	2.89	4.54	.99
11		14.1	8.4	7.5	7.4	7.76	1.51	.98	1.63
13		7.9	6.1	6.3	6.7	3.63	.85	.95	.80
20		7.8	6.3	---	4.8	2.34	3.40	---	.22
23		11.1	6.0	6.1	8.0	5.33	1.80	1.28	1.20
4	ZDLA	15.7	13.7	12.6	10.2	5.18	5.89	5.54	1.43
8		8.1	14.2	---	3.9	1.22	6.39	---	.98
9		14.6	18.2	9.9	18.8	11.39	11.28	3.27	13.16
14		14.2	10.3	12.0	6.9	4.40	2.99	4.08	1.38
19		13.4	5.9	6.3	8.2	2.41	.53	---	.21
24		14.5	9.8	11.4	8.1	3.63	2.55	4.45	1.38
2	ZALA	15.5	17.4	9.7	13.1	5.74	4.52	3.10	1.70
5		11.8	8.8	7.2	9.2	5.07	1.49	2.23	1.47
12		14.0	9.8	13.2	---	6.72	4.02	.79	---
16		14.6	8.9	6.2	9.9	12.18	1.78	1.30	3.07
18		16.8	9.2	6.8	8.7	11.42	2.58	1.36	1.74
22		13.6	7.3	7.1	7.5	7.34	1.75	2.63	2.78

[a] -- indicates missing value.

Table C-8

Platelet Counts and Mean Platelet Volumes of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Platelet Count Experimental Day				Mean Platelet Volume Experimental Day			
		3	13	23	33	3	13	23	33
		Count ($10^3/mm^3$)				fL			
1	ZDLD	906	1178	---[a]	1054	5.9	6.6	---	6.4
6		498	---	---	1011	6.9	---	---	7.0
10		411	1464	800	657	6.6	7.0	6.9	6.8
15		615	945	664	---	7.9	7.5	7.5	---
17		982	1406	895	1268	7.6	7.2	7.1	7.2
21		939	1264	978	---	7.2	6.8	6.5	---
3	ZALD	816	1152	1105	954	6.9	7.3	7.2	6.9
7		---	970	1078	930	---	7.4	7.3	7.0
11		695	1144	807	715	7.5	6.9	6.7	6.6
13		1046	847	689	906	7.4	7.3	7.1	6.6
20		1415	1242	899	1038	7.5	6.7	6.7	6.8
23		1101	1290	830	1109	7.1	6.8	6.9	6.6
4	ZDLA	571	517	---	761	6.9	7.0	---	6.8
8		807	435	---	749	6.9	7.5	---	6.6
9		907	1578	---	958	7.4	7.4	---	6.7
14		1442	1422	792	340	7.1	6.9	6.3	6.1
19		1188	970	731	1117	7.0	6.6	6.6	6.5
24		1442	957	831	422	7.6	6.8	6.7	6.5
2	ZALA	562	627	863	869	7.0	7.2	6.6	6.5
5		679	772	848	---	6.9	7.1	6.7	---
12		935	903	912	---	7.3	7.5	7.2	---
16		994	908	731	612	7.1	7.1	6.6	6.7
18		937	967	864	1095	7.5	6.4	6.2	6.2
22		1046	1202	823	912	7.3	6.6	6.7	6.6

[a] indicates a missing value.

Table C-9

Leukocyte Alkaline Phosphatase (LAP) Score and Bleeding Time of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	LAP Score Experimental Day				Bleeding Time Experimental Day			
		3	13	23	33	2	12	22	32
		Score (33 cells)				Seconds			
1	ZDLD	14	6	29	39	127	55	40	35
6		35	35	11	40	85	35	43	28
10		26	32	25	39	65	30	50	25
15		40	30	41	--[a]	40	55	25	20
17		20	44	18	52	52	20	25	32
21		23	36	34	21	50	50	20	24
3	ZALD	34	35	34	32	118	56	30	35
7		22	40	23	36	65	25	50	70
11		18	23	20	32	45	35	45	38
13		24	8	21	18	75	55	70	33
20		20	34	14	40	65	30	20	40
23		42	31	23	38	55	50	25	15
4	ZDLA	31	28	22	45	65	80	80	50
8		41	35	29	34	65	30	65	35
9		39	33	33	37	62	35	60	40
14		38	19	32	40	45	38	45	25
19		31	39	29	36	55	25	50	50
24		68	31	37	36	45	55	25	25
2	ZALA	36	26	33	31	64	70	25	20
5		38	37	27	27	105	40	40	15
12		29	47	12	48	40	50	35	30
16		30	31	31	45	50	45	65	35
18		45	42	23	41	90	40	30	20
22		44	29	24	33	65	60	35	50

[a] indicates a missing value.

Table C-10

Plasma Fatty Acid Concentration of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Fatty Acid Concentration							
		Myristic	Palmitic	Stearic	Oleic	Linoleic	Eicosa-trienoic[a]	Aracridonic	Ratio[b]
		mg/dl							
1	ZDLD	3.406	49.014	16.487	18.970	0.461	1.167	2.116	74.809
6		7.790	43.836	11.146	17.739	0.644	0.302	0.737	54.050
10		2.992	74.917	23.303	71.493	4.315	3.452	23.648	42.000
17		2.701	67.654	27.073	68.573	4.023	2.557	14.686	50.056
21		1.997	43.038	17.083	35.816	2.987	3.116	13.545	42.166
3	ZALD	2.621	51.394	19.886	50.950	2.244	0.711	10.065	15.696
7		3.010	46.305	22.015	29.750	1.610	0.350	5.460	11.217
11		1.764	53.096	20.308	50.450	3.792	0.859	17.353	10.926
13		1.778	61.036	22.012	24.002	0.933	0.704	4.367	28.403
20		2.420	43.622	17.238	33.404	2.265	1.314	6.120	37.119
23		1.609	42.687	13.373	33.553	2.773	0.940	11.269	13.302
4	ZDLA	3.747	58.374	19.948	50.926	17.718	1.755	31.737	13.118
8		---[c]	---	---	---	---	---	---	---
9		1.965	69.825	16.660	37.636	19.352	1.217	36.589	7.107
14		1.584	58.249	24.752	33.813	17.677	1.710	37.023	9.758
19		2.687	82.482	26.388	54.721	28.532	1.629	36.852	12.642
24		---	---	---	---	---	---	---	---
2	ZALA	2.444	55.195	21.155	27.643	12.590	1.433	28.525	9.237
5		2.356	56.823	17.189	15.066	4.836	---	---	---
12		2.922	65.087	18.798	50.307	20.721	1.436	39.520	8.851
16		1.835	55.171	23.439	48.783	23.624	1.765	47.482	8.637
18		1.174	48.475	16.401	13.793	2.867	---	5.660	---
22		2.414	58.963	23.383	45.485	23.654	1.897	50.462	9.264

a 5,8,11 Eiocosatrienoic Acid.
b Ratio = (Eicosatrienoic Acid/Arachidonic Acid)*100.
c indicates a missing value.

Table C-11

Serum Zinc and Testicular Copper, Iron, Zinc and Manganese Concentrations of Young Male Rats Fed Zinc and Linoleic Acid.

Cage	Diet	Plasma	Testicular			
		Zinc	Copper	Iron	Zinc	Manganese
		ppm	mcg/g dry wt.			
1	ZDLD	213	12.8	114	144	2.4
6		140	10.0	110	175	2.5
10		180	21.9	224	127	5.8
15		--[a]	17.7	114	118	4.4
17		100	13.3	113	133	2.4
21		100	15.6	140	138	3.5
3	ZALD	200	11.7	101	184	2.5
7		140	9.4	100	177	2.4
11		160	9.6	102	169	2.4
13		170	11.2	102	177	1.9
20		210	8.9	93	180	2.5
23		140	11.0	105	168	2.3
4	ZDLA	100	13.4	112	142	3.1
8		---	12.3	196	123	6.7
9		90	18.1	132	217	4.5
14		110	21.4	237	120	5.4
19		110	21.8	188	243	5.1
24		---	16.7	173	126	7.3
2	ZALA	160	13.6	103	176	2.3
5		163	11.5	102	187	2.4
12		130	13.2	139	167	2.8
16		150	12.5	104	164	2.0
18		160	9.8	99	169	2.6
22		150	12.2	106	167	2.6

[a] indicates a missing value.

Appendix D

Univariate Statistical Analyses

Table D-1

Univariate Analysis of Variance For Plasma Zinc Concentration of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Serum Zinc					
	Model	3	0.112	4.18	0.02
	Zinc	1	0.006	7.60	0.01
	Lino[1]	1	0.004	5.46	0.03
	Zinc*Lino	1	0.001	0.97	0.34
	Error	11	0.015		
	Total	14	0.026		

[1] Linoleic Acid

Table D-2

Univariate Analysis of Variance For Plasma Ratio of Eicosatrienoic Acid to Arachidonic Acid In Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Ratio of Eicosatrienoic Acid to Arachidonic Acid					
	Model	3	.2195	7.51	0.003
	Zinc	1	.0459	4.86	0.04
	Lino	1	.1216	12.87	0.003
	Zinc*Lino	1	.0420	4.44	0.05
	Error	15	.1418		
	Total	18	.3545		

Appendix E

Multivariate Statistical Analyses

Table E-1

Multivariate Analysis of Variance for Plasma Fatty Acid Profile of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Myristic					
	Model	3	7.81	1.50	0.26
	Zinc	1	3.21	1.85	0.19
	Lino	1	1.43	0.77	0.39
	Zinc*Lino	1	2.54	1.46	0.24
	Error	15	26.09		
	Total	18	33.90		
Palmitic					
	Model	3	757.79	2.42	0.11
	Zinc	1	246.98	2.60	0.14
	Lino	1	482.77	4.63	0.05
	Zinc*Lino	1	7.96	0.08	0.78
	Error	15	1562.51		
	Total	18	2320.30		
Stearic					
	Model	3	34.71	0.61	0.61
	Zinc	1	0.02	0.00	0.98
	Lino	1	34.57	1.82	0.20
	Zinc*Lino	1	0.15	0.10	0.93
	Error	15	284.78		
	Total	18	482.76		
Oleic					
	Model	3	165.09	0.21	0.89
	Zinc	1	52.11	0.20	0.66
	Lino	1	70.05	0.26	0.61
	Zinc*Lino	1	21.14	0.08	0.78
	Error	15	3989.36		
	Total	18	4154.45		
Linoleic					
	Model	3	1521.02	41.97	0.001
	Zinc	1	0.91	0.08	0.79
	Lino	1	1513.81	125.31	0.001
	Zinc*Lino	1	0.23	0.02	0.89
	Error	15	181.21		
	Total	18	1702.23		
Eicosatrienoic					
	Model	3	4.84	3.02	0.06
	Zinc	1	1.81	3.38	0.09
	Lino	1	0.08	0.17	0.69
	Zinc*Lino	1	2.13	3.99	0.06
	Error	15	8.02		
	Total	18	12.86		
Arachidonic					
	Model	3	3863.47	24.44	0.001
	Zinc	1	19.45	0.37	0.55
	Lino	1	3748.23	71.13	0.001
	Zinc*Lino	1	69.98	1.33	0.27
	Error	15	790.42		
	Total	18			

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Zinc Effect	3.12	7,9	0.058
No overall Lino Effect	20.27	7,9	0.001
No overall Zinc*Lino Effect	2.71	7,9	0.083

Table E-2

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on Weight Change of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	8.84	0.88	0.47
	Zinc	1	1.83	0.54	0.47
	Lino	1	3.05	0.91	0.35
	Zinc*Lino	1	3.96	1.81	0.29
	Error	20	67.21		
	Total	23	76.05		
Linear Effect Over Time					
	Model	3	21.05	9.91	0.001
	Zinc	1	20.69	29.22	0.001
	Lino	1	0.34	0.49	0.49
	Zinc*Lino	1	0.01	0.01	0.92
	Error	20	14.17		
	Total	23	35.21		
Quadratic Effect Over Time					
	Model	3	1.95	10.92	0.001
	Zinc	1	1.86	31.40	0.001
	Lino	1	0.06	1.01	0.32
	Zinc*Lino	1	0.02	0.36	0.56
	Error	20	1.19		
	Total	23	3.13		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	20.70	3,18	0.001
No Overall Lino Effect	0.79	3,18	0.51
No Overall Zinc*Lino Effect	0.93	3,18	0.44

Table E-3

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on Food Intake of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	66.97	4.01	0.022
	Zinc	1	60.36	10.85	0.004
	Lino	1	3.32	0.60	0.44
	Zinc*Lino	1	3.28	0.59	0.45
	Error	20	111.34		
	Total	23	178.35		
Linear Effect Over Time					
	Model	3	13.01	12.36	0.001
	Zinc	1	9.71	27.65	0.001
	Lino	1	1.32	3.78	0.066
	Zinc*Lino	1	1.98	5.66	0.027
	Error	20	7.01		
	Total	23	20.04		
Quadratic Effect Over Time					
	Model	3	2.27	4.94	0.01
	Zinc	1	1.31	8.54	0.008
	Lino	1	0.40	2.66	0.12
	Zinc*Lino	1	0.55	3.60	0.07
	Error	20	3.06		
	Total	23	5.39		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	15.60	3,18	0.001
No Overall Lino Effect	1.17	3,18	0.35
No Overall Zinc*Lino Effect	1.72	3,18	0.20

Table E-4

Multivariate Analysis of Variance for **Appearance/Muscle Tone and Dermal Lesions** on Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Appearance/Muscle Tonus For Days 18-33					
	Model	3	12.57	1.92	0.17
	Zinc	1	6.13	2.81	0.12
	Lino	1	0.04	0.02	0.88
	Zinc*Lino	1	0.74	0.34	0.57
	Error	14	30.50		
	Total	17	43.11		
Dermal Lesions For Days 26-33					
	Model	3	162.10	7.03	0.004
	Zinc	1	34.43	4.48	0.05
	Lino	1	39.00	5.08	0.04
	Zinc*Lino	1	15.87	2.07	0.17
	Error	20	107.53		
	Total	23	269.61		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Zinc Effect	3.65	2,13	0.05
No overall Lino Effect	2.40	2,13	0.13
No overall Zinc*Lino Effect	1.18	2,13	0.34

Table E-5

Multivariate Analysis of Variance for Testicular Mineral Concentration of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Copper					
	Model	3	174.9	6.41	0.003
	Zinc	1	152.4	16.74	0.001
	Lino	1	22.5	2.47	0.13
	Zinc*Lino	1	0.1	0.01	0.92
	Error	20	182.0		
	Total	23	356.9		
Iron					
	Model	3	117387.2	2.29	0.11
	Zinc	1	72644.0	4.25	0.05
	Lino	1	25363.0	1.48	0.24
	Zinc*Lino	1	19380.2	1.13	0.30
	Error	20	341825.1		
	Total	23	459212.3		
Zinc					
	Model	3	4801.0	1.86	0.16
	Zinc	1	3210.9	0.01	0.07
	Lino	1	520.8	0.61	0.45
	Zinc*Lino	1	1069.3	1.24	0.28
	Error	20	17229.1		
	Total	23	22030.1		
Manganese					
	Model	3	33.59	11.03	0.001
	Zinc	1	23.96	23.60	0.001
	Lino	1	5.40	5.32	0.03
	Zinc*Lino	1	4.23	4.17	0.05
	Error	20	20.30		
	Total	23	53.89		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Zinc Effect	6.54	4,17	0.002
No overall Lino Effect	1.72	4,17	0.19
No overall Zinc*Lino Effect	2.36	4,17	0.09

Table E-6

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on the Total Leukocyte Count of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	70.91	3.28	0.042
	Zinc	1	68.78	9.54	0.005
	Lino	1	0.42	0.06	0.81
	Zinc*Lino	1	1.71	0.24	0.63
	Error	20	144.26		
	Total	23	215.18		
Linear Effect Over Time					
	Model	3	5.04	1.48	0.25
	Zinc	1	2.53	2.23	0.15
	Lino	1	0.97	0.86	0.37
	Zinc*Lino	1	1.53	1.35	0.25
	Error	20	22.69		
	Total	23	27.72		
Quadratic Effect Over Time					
	Model	3	9.32	2.24	0.12
	Zinc	1	7.25	5.23	0.03
	Lino	1	0.01	0.01	0.94
	Zinc*Lino	1	2.07	1.49	0.23
	Error	20	27.74		
	Total	23	37.07		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	3.70	3,18	0.03
No Overall Lino Effect	0.29	3,18	0.83
No Overall Zinc*Lino Effect	1.24	3,18	0.32

Table E-7

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on the Absolute Granulocyte Count of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	3351.26	3.80	0.03
	Zinc	1	3346.08	11.40	0.003
	Lino	1	0.24	0.00	0.97
	Zinc*Lino	1	4.94	0.02	0.90
	Error	20	5872.66		
	Total	23	9223.93		
Linear Effect Over Time					
	Model	3	1358.14	2.71	0.07
	Zinc	1	975.48	5.84	0.03
	Lino	1	370.02	2.22	0.15
	Zinc*Lino	1	12.64	0.08	0.78
	Error	20	3340.74		
	Total	23	4698.88		
Quadratic Effect Over Time					
	Model	3	729.91	1.01	0.41
	Zinc	1	379.37	1.57	0.22
	Lino	1	45.60	0.19	0.67
	Zinc*Lino	1	304.94	1.26	0.27
	Error	20	4833.85		
	Total	23	5563.75		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	5.51	3,18	0.007
No Overall Lino Effect	1.13	3,18	0.36
No Overall Zinc*Lino Effect	0.67	3,18	0.58

Table E-8

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on Platelet Counts of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	161317.5	0.77	0.52
	Zinc	1	13701.3	0.20	0.66
	Lino	1	146904.6	2.10	0.16
	Zinc*Lino	1	711.6	0.01	0.92
	Error	20	1402049.2		
	Total	23	1563366.7		
Linear Effect Over Time					
	Model	3	46746.8	0.70	0.56
	Zinc	1	32259.3	1.46	0.24
	Lino	1	2589.6	0.12	0.74
	Zinc*Lino	1	11897.8	0.54	0.47
	Error	20	442578.4		
	Total	23	489325.2		
Quadratic Effect Over Time					
	Model	3	120754.7	2.29	0.11
	Zinc	1	33581.5	1.91	0.18
	Lino	1	59581.1	3.41	0.08
	Zinc*Lino	1	27298.4	1.55	0.22
	Error	20	351291.9		
	Total	23	472046.6		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	0.95	3,18	0.43
No Overall Lino Effect	1.24	3,18	0.32
No Overall Zinc*Lino Effect	1.19	3,18	0.34

Table E-9

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on Mean Platelet Volume of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	0.102	0.26	0.85
	Zinc	1	0.006	0.05	0.83
	Lino	1	0.061	0.47	0.50
	Zinc*Lino	1	0.035	0.27	0.61
	Error	20	2.579		
	Total	23	2.681		
Linear Effect Over Time					
	Model	3	0.148	2.61	0.08
	Zinc	1	0.045	2.36	0.14
	Lino	1	0.088	4.62	0.04
	Zinc*Lino	1	0.016	0.85	0.37
	Error	20	0.379		
	Total	23	0.528		
Quadratic Effect Over Time					
	Model	3	0.009	0.11	0.96
	Zinc	1	0.003	0.09	0.76
	Lino	1	0.004	0.13	0.72
	Zinc*Lino	1	0.003	0.10	0.75
	Error	20	0.622		
	Total	23	0.632		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	0.82	3,18	0.49
No Overall Lino Effect	2.01	3,18	0.15
No Overall Zinc*Lino Effect	0.66	3,18	0.58

Table E-10

Multivariate Measures Analysis of Variance of the Effects of Zinc and Linoleic Acid Over **Time** on **Leukocyte Alkaline Phosphatase** in Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	77.5	0.73	0.54
	Zinc	1	11.4	0.32	0.57
	Lino	1	57.2	1.61	0.22
	Zinc*Lino	1	8.8	0.25	0.62
	Error	20	709.2		
	Total	23	786.7		
Linear Effect Over Time					
	Model	3	137.18	2.77	0.69
	Zinc	1	18.55	1.12	0.30
	Lino	1	96.40	5.83	0.03
	Zinc*Lino	1	22.23	1.34	0.25
	Error	20	330.65		
	Total	23	467.8		
Quadratic Effects Over Time					
	Model	3	18.34	0.42	0.73
	Zinc	1	7.87	0.54	0.46
	Lino	1	10.33	0.71	0.41
	Zinc*Lino	1	0.12	0.01	0.92
	Error	20	290.28		
	Total	23	308.62		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	0.87	3,18	0.47
No Overall Lino Effect	2.91	3,18	0.06
No Overall Zinc*Lino Effect	0.60	3,18	0.62

Table E-11

Multivariate Analysis of Variance of the Effects of Zinc and Linoleic Acid Over Time on Bleeding Time in Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Intercept					
	Model	3	610.30	1.32	0.29
	Zinc	1	5.45	0.04	0.85
	Lino	1	496.74	3.22	0.09
	Zinc*Lino	1	108.10	0.70	0.41
	Error	20	3084.22		
	Total	23	3694.52		
Linear Effect Over Time					
	Model	3	317.89	1.39	0.27
	Zinc	1	40.82	0.53	0.47
	Lino	1	40.82	0.53	0.47
	Zinc*Lino	1	236.25	3.09	0.09
	Error	20	1528.51		
	Total	23	1846.4		
Quadratic Effects Over Time					
	Model	3	230.32	1.29	0.31
	Zinc	1	27.62	0.46	0.50
	Lino	1	196.94	3.31	0.08
	Zinc*Lino	1	5.75	0.10	0.75
	Error	20	1190.53		
	Total	23	1420.85		

Multivariate Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No Overall Zinc Effect	0.22	3,18	0.87
No Overall Lino Effect	1.26	3,18	0.32
No Overall Zinc*Lino Effect	1.64	3,18	0.22

Appendix F

Repeated Measures Statistical Analyses

Table F-1

Repeated Measures Analysis of Variance for Weight Change of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Weight Change For Days 2-5					
	Model	3	1188.0	8.27	0.001
	Zinc	1	1120.6	23.41	0.001
	Lino	1	0.7	0.01	0.91
	Zinc*Lino	1	66.6	1.39	0.25
	Error	20	975.3		
	Total	23	2145.3		
Weight Change For Days 6-9					
	Model	3	268.2	1.57	0.22
	Zinc	1	170.7	3.01	0.09
	Lino	1	96.0	1.69	0.21
	Zinc*Lino	1	1.5	0.03	0.87
	Error	20	1097.7		
	Total	23	3464.5		
Weight Change For Days 10-13					
	Model	3	365.8	1.88	0.16
	Zinc	1	165.4	2.55	0.12
	Lino	1	108.4	1.67	0.21
	Zinc*Lino	1	92.0	1.42	0.24
	Error	20	1296.2		
	Total	23	1661.9		
Weight Change For Days 14-17					
	Model	3	134.0	1.16	0.35
	Zinc	1	42.7	1.10	0.31
	Lino	1	10.7	0.28	0.61
	Zinc*Lino	1	80.7	2.09	0.16
	Error	20	773.3		
	Total	23	907.3		

Table F-1 (Continued).

Repeated Measures Analysis of Variance for Weight Change.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Weight Change For Days 18-21					
	Model	3	473.8	5.35	0.007
	Zinc	1	459.4	15.57	0.001
	Lino	1	9.4	0.32	0.58
	Zinc*Lino	1	5.0	0.17	0.68
	Error	20	590.2		
	Total	23	1063.9		
Weight Change For Days 22-25					
	Model	3	129.1	2.08	0.13
	Zinc	1	126.0	6.10	0.02
	Lino	1	2.0	0.10	0.76
	Zinc*Lino	1	1.0	0.05	0.82
	Error	20	413.5		
	Total	23	542.6		
Weight Change For Days 26-29					
	Model	3	206.7	2.10	0.13
	Zinc	1	54.0	1.64	0.21
	Lino	1	2.7	0.08	0.78
	Zinc*Lino	1	150.0	4.57	0.04
	Error	20	656.7		
	Total	23	863.3		
Weight Change For Days 30-33					
	Model	3	9.5	0.20	0.89
	Zinc	1	3.4	0.21	0.65
	Lino	1	1.0	0.07	0.80
	Zinc*Lino	1	5.0	0.32	0.57
	Error	20	316.5		
	Total	23	325.9		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	53.0	7,14	0.001
No overall Time*Zinc Effect	17.3	7,14	0.001
No overall Time*Lino Effect	0.9	7,14	0.56
No overall Time*Zinc*Lino Effect	1.1	7,14	0.41

Table F-2

Repeated Measures Analysis of Variance for Food Intake of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Food Intake For Days 2-5					
	Model	3	1818.8	6.85	0.002
	Zinc	1	1350.0	15.26	0.001
	Lino	1	228.2	2.58	0.12
	Zinc*Lino	1	240.7	2.72	0.11
	Error	20	1769.0		
	Total	23	3587.8		
Food Intake For Days 6-9					
	Model	3	209.1	3.82	0.026
	Zinc	1	187.0	10.25	0.005
	Lino	1	0.0	0.00	0.96
	Zinc*Lino	1	22.0	1.21	0.28
	Error	20	364.8		
	Total	23	573.9		
Food Intake For Days 10-13					
	Model	3	521.1	12.11	0.001
	Zinc	1	477.0	33.26	0.001
	Lino	1	22.0	1.54	0.23
	Zinc*Lino	1	22.0	1.54	0.23
	Error	20	286.8		
	Total	23	807.9		
Food Intake For Days 14-17					
	Model	3	134.8	2.78	0.07
	Zinc	1	121.5	7.52	0.02
	Lino	1	10.6	0.66	0.42
	Zinc*Lino	1	2.7	0.17	0.68
	Error	20	323.0		
	Total	23	457.8		

Table F-2 (Continued).

Repeated Measures Analysis of Variance of Food Intake.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Food Intake For Days 18-21					
	Model	3	8.5	0.19	0.90
	Zinc	1	8.1	0.53	0.47
	Lino	1	0.2	0.01	0.91
	Zinc*Lino	1	0.2	0.01	0.91
	Error	20	305.3		
	Total	23	313.8		
Food Intake For Days 22-25					
	Model	3	58.5	1.69	0.20
	Zinc	1	57.0	4.94	0.04
	Lino	1	1.0	0.09	0.76
	Zinc*Lino	1	0.4	0.03	0.85
	Error	20	231.2		
	Total	23	289.6		
Food Intake For Days 26-29					
	Model	3	308.5	4.58	0.014
	Zinc	1	308.2	13.72	0.001
	Lino	1	0.2	0.01	0.93
	Zinc*Lino	1	0.2	0.01	0.93
	Error	20	449.3		
	Total	23	757.8		
Food Intake For Days 30-33					
	Model	3	22.1	2.06	0.13
	Zinc	1	22.0	6.17	0.02
	Lino	1	0.0	0.01	0.91
	Zinc*Lino	1	0.0	0.01	0.91
	Error	20	71.5		
	Total	23	93.6		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	105.4	7,14	0.001
No overall Time*Zinc Effect	14.5	7,14	0.001
No overall Time*Lino Effect	0.7	7,14	0.65
No overall Time*Zinc*Lino Effect	1.9	7,14	0.15

Table F-3

Repeated Measures Analysis of Variance for Food Efficiency Ratio of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
FER For Days 2-5					
	Model	3	2543.8	3.90	0.02
	Zinc	1	2371.5	10.91	0.004
	Lino	1	0.5	0.00	0.96
	Zinc*Lino	1	171.8	0.79	0.38
	Error	20	4346.1		
	Total	23	6889.9		
FER For Days 6-9					
	Model	3	1056.8	1.54	0.23
	Zinc	1	569.4	2.48	0.13
	Lino	1	465.9	2.03	0.17
	Zinc*Lino	1	21.5	0.09	0.76
	Error	20	4586.0		
	Total	23	5642.8		
FER For Days 10-13					
	Model	3	1854.4	1.79	0.18
	Zinc	1	404.8	1.17	0.29
	Lino	1	783.8	2.27	0.15
	Zinc*Lino	1	665.7	1.93	0.18
	Error	20	6910.3		
	Total	23	8764.7		
FER For Days 14-17					
	Model	3	564.2	1.00	0.41
	Zinc	1	113.7	0.60	0.44
	Lino	1	49.2	0.26	0.61
	Zinc*Lino	1	401.4	2.13	0.16
	Error	20	3770.7		
	Total	23	4334.9		

Table F-3 (Continued).

Repeated Measures Analysis of Variance for Food Efficiency Ratio.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
FER For Days 18-21					
	Model	3	2922.9	4.95	0.01
	Zinc	1	2853.5	14.49	0.001
	Lino	1	34.6	0.18	0.68
	Zinc*Lino	1	34.6	0.18	0.68
	Error	20	3939.6		
	Total	23	6862.6		
FER For Days 22-25					
	Model	3	596.2	1.83	0.17
	Zinc	1	581.2	5.36	0.03
	Lino	1	11.4	0.11	0.75
	Zinc*Lino	1	3.5	0.03	0.85
	Error	20	2167.0		
	Total	23	2763.2		
FER For Days 26-29					
	Model	3	1235.1	2.36	0.10
	Zinc	1	296.2	1.70	0.20
	Lino	1	1.2	0.01	0.93
	Zinc*Lino	1	937.7	5.38	0.03
	Error	20	3487.5		
	Total	23	4722.7		
FER For Days 30-33					
	Model	3	57.7	0.19	0.90
	Zinc	1	17.3	0.17	0.68
	Lino	1	7.7	0.08	0.78
	Zinc*Lino	1	32.7	0.35	0.57
	Error	20	2002.6		
	Total	23	2060.3		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	33.5	7,14	0.001
No overall Time*Zinc Effect	13.4	7,14	0.001
No overall Time*Lino Effect	0.9	7,14	0.49
No overall Time*Zinc*Lino Effect	1.2	7,14	0.35

Table F-4

Repeated Measures Analysis of Variance for Total Leukocyte Count of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Total Leukocyte Count On Day 3					
	Model	3	4.5	0.18	0.91
	Zinc	1	0.0	0.00	0.98
	Lino	1	4.4	0.53	0.47
	Zinc*Lino	1	0.0	0.00	0.97
	Error	16	137.4		
	Total	19	141.9		
Total Leukocyte Count On Day 13					
	Model	3	15.3	0.32	0.81
	Zinc	1	13.9	0.87	0.36
	Lino	1	0.5	0.03	0.85
	Zinc*Lino	1	0.8	0.05	0.82
	Error	16	256.2		
	Total	19	271.5		
Total Leukocyte Count On Day 23					
	Model	3	54.5	2.48	0.10
	Zinc	1	53.8	7.33	0.02
	Lino	1	0.4	0.05	0.82
	Zinc*Lino	1	0.3	0.04	0.84
	Error	16	117.4		
	Total	19	171.8		
Total Leukocyte Count On Day 33					
	Model	3	19.2	0.65	0.59
	Zinc	1	14.6	1.49	0.23
	Lino	1	0.1	0.01	0.92
	Zinc*Lino	1	4.5	0.46	0.50
	Error	16	156.5		
	Total	19	175.7		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freeedom	p-value
No overall Time Effect	16.70	3,14	0.001
No overall Time*Zinc Effect	1.84	3,14	0.19
No overall Time*Lino Effect	0.23	3,14	0.87
No overall Time*Zinc*Lino Effect	0.15	3,14	0.92

Table F-5

Repeated Measures Analysis of Variance for Absolute Granulocyte Count of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Absolute Granulocyte Count On Day 3					
	Model	3	3541.9	1.61	0.23
	Zinc	1	325.2	3.30	0.52
	Lino	1	2098.4	2.85	0.11
	Zinc*Lino	1	791.0	1.08	0.32
	Error	14	10296.7		
	Total	17	13838.7		
Absolute Granulocyte Count On Day 13					
	Model	3	3531.6	2.08	0.14
	Zinc	1	3256.7	5.77	0.03
	Lino	1	143.1	0.25	0.62
	Zinc*Lino	1	132.0	0.23	0.64
	Error	14	7906.1		
	Total	17	11437.7		
Absolute Granulocyte Count On Day 23					
	Model	3	3767.4	2.08	0.15
	Zinc	1	3578.3	5.94	0.03
	Lino	1	13.6	0.02	0.88
	Zinc*Lino	1	175.4	0.29	0.59
	Error	14	8439.7		
	Total	17	12207.1		
Absolute Granulocyte Count On Day 33					
	Model	3	2580.1	1.11	0.37
	Zinc	1	647.6	0.83	0.37
	Lino	1	1507.9	1.94	0.19
	Zinc*Lino	1	424.5	0.55	0.47
	Error	14	10863.4		
	Total	17	13443.5		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	17.2	3,12	0.001
No overall Time*Zinc Effect	2.4	3,12	0.12
No overall Time*Lino Effect	0.9	3,12	0.47
No overall Time*Zinc*Lino Effect	1.4	3,12	0.29

Table F-6

Repeated Measures Analysis of Variance for Platelet Count of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Platelet Count On Day 3					
	Model	3	621058.4	3.13	0.07
	Zinc	1	18596.1	0.28	0.61
	Lino	1	219696.1	3.32	0.09
	Zinc*Lino	1	487153.4	7.37	0.02
	Error	10	661223.1		
	Total	13	1282281.5		
Platelet Count On Day 13					
	Model	3	349679.0	2.72	0.10
	Zinc	1	187345.5	4.37	0.06
	Lino	1	216960.1	5.07	0.05
	Zinc*Lino	1	9371.5	0.22	0.65
	Error	10	428284.7		
	Total	13	777963.7		
Platelet Count On Day 23					
	Model	3	13523.8	0.39	0.76
	Zinc	1	4134.9	0.36	0.56
	Lino	1	9161.5	0.79	0.39
	Zinc*Lino	1	227.4	0.02	0.89
	Error	10	115623.9		
	Total	13	129147.7		
Platelet Count On Day 33					
	Model	3	220957.4	0.65	0.59
	Zinc	1	73699.4	0.97	0.35
	Lino	1	93045.4	1.22	0.29
	Zinc*Lino	1	54212.6	0.71	0.41
	Error	10	760136.4		
	Total	13	981093.7		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	7.96	3,8	0.009
No overall Time*Zinc Effect	1.41	3,8	0.308
No overall Time*Lino Effect	5.17	3,8	0.028
No overall Time*Zinc*Lino Effect	3.80	3,8	0.058

Table F-7

Repeated Measures Analysis of Variance for Mean Platelet Volume of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Mean Platelet Volume On Day 3					
	Model	3	0.05	0.14	0.93
	Zinc	1	0.02	0.20	0.66
	Lino	1	0.00	0.04	0.84
	Zinc*Lino	1	0.03	0.24	0.63
	Error	10	1.19		
	Total	13	1.19		
Mean Platelet Volume On Day 13					
	Model	3	0.20	0.81	0.52
	Zinc	1	0.00	0.02	0.90
	Lino	1	0.20	2.41	0.15
	Zinc*Lino	1	0.02	0.23	0.63
	Error	10	0.83		
	Total	13	1.04		
Mean Platelet Volume On Day 23					
	Model	3	0.61	4.40	0.03
	Zinc	1	0.00	0.13	0.72
	Lino	1	0.58	12.52	0.005
	Zinc*Lino	1	0.00	0.09	0.77
	Error	10	0.46		
	Total	13	1.07		
Mean Platelet Volume On Day 33					
	Model	3	0.57	4.68	0.03
	Zinc	1	0.02	0.53	0.48
	Lino	1	0.54	13.31	0.005
	Zinc*Lino	1	0.15	3.60	0.09
	Error	10	0.41		
	Total	13	0.98		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	11.90	3,8	0.003
No overall Time*Zinc Effect	0.26	3,8	0.85
No overall Time*Lino Effect	1.73	3,8	0.24
No overall Time*Zinc*Lino Effect	1.92	3,8	0.21

Table F-8

Repeated Measures Analysis of Variance for Leukocyte Alkaline Phosphatase of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Leukocyte Alkaline Phosphatase On Day 3					
	Model	3	1189.9	4.08	0.02
	Zinc	1	2.3	0.02	0.88
	Lino	1	1125.3	11.57	0.003
	Zinc*Lino	1	78.2	0.80	0.38
	Error	19	1847.9		
	Total	22	3037.7		
Leukocyte Alkaline Phosphatase On Day 13					
	Model	3	148.4	0.46	0.72
	Zinc	1	8.2	0.08	0.78
	Lino	1	71.3	0.66	0.43
	Zinc*Lino	1	62.2	0.57	0.45
	Error	19	2062.9		
	Total	22	2211.2		
Leukocyte Alkaline Phosphatase On Day 23					
	Model	3	217.4	1.45	0.26
	Zinc	1	55.5	1.11	0.31
	Lino	1	127.1	2.55	0.12
	Zinc*Lino	1	28.1	0.56	0.46
	Error	19	948.0		
	Total	22	1165.5		
Leukocyte Alkaline Phosphatase On Day 33					
	Model	3	122.1	0.63	0.61
	Zinc	1	52.0	0.80	0.38
	Lino	1	30.7	0.47	0.50
	Zinc*Lino	1	36.2	0.56	0.46
	Error	19	1229.6		
	Total	22	1351.7		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	6.77	3,17	0.003
No overall Time*Zinc Effect	0.33	3,17	0.81
No overall Time*Lino Effect	1.87	3,17	0.17
No overall Time*Zinc*Lino Effect	0.51	3,17	0.68

Table F-9

Repeated Measures Analysis of Variance for Bleeding Time of Young Male Rats Fed Zinc and Linoleic Acid.

Dependent Variable	Source	Degrees of Freedom	Type III Sum of Squares	F-value	Probability Greater than F-value
Bleeding Time On Day 2					
	Model	3	840.5	0.48	0.70
	Zinc	1	273.4	0.46	0.50
	Lino	1	345.0	0.59	0.45
	Zinc*Lino	1	222.0	0.38	0.54
	Error	20	11771.2		
	Total	23	12611.6		
Bleeding Time On Day 12					
	Model	3	366.0	0.51	0.68
	Zinc	1	96.7	0.40	0.53
	Lino	1	216.0	0.90	0.35
	Zinc*Lino	1	54.0	0.23	0.64
	Error	20	4793.3		
	Total	23	5159.3		
Bleeding Time On Day 22					
	Model	3	1388.8	1.77	0.19
	Zinc	1	140.2	0.54	0.47
	Lino	1	522.7	2.00	0.17
	Zinc*Lino	1	726.0	2.77	0.11
	Error	20	5235.0		
	Total	23	6623.8		
Bleeding Time On Day 32					
	Model	3	626.2	1.30	0.30
	Zinc	1	6.0	0.04	0.85
	Lino	1	0.0	0.00	1.00
	Zinc*Lino	1	620.2	3.86	0.06
	Error	20	3211.7		
	Total	23	3837.8		

Repeated Measures Analysis of Variance Results Based on Roy's Maximum Root Criterion:

Hypothesis	Roy's Maximum Root	Degrees of Freedom	p-value
No overall Time Effect	11.17	3,18	0.002
No overall Time*Zinc Effect	0.58	3,18	0.68
No overall Time*Lino Effect	1.14	3,18	0.36
No overall Time*Zinc*Lino Effect	1.19	3,18	0.34